BIONANOTECHNOLOGY: NEXT-GENERATION THERAPEUTIC TOOLS

Editors:
Alaa A. Aljabali
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Bionanotechnology: Next-Generation Therapeutic Tools

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PREFACE

This book describes the design and characterization of bionanomaterials, which exhibit distinctive physical, chemical, and biological properties, and discusses how these functional nanomaterials enable the precise manipulation of the architectural, physical, and biochemical cellular environment *in vitro* and *in vivo*. Besides, it covers how they can act as carriers of diagnostic or therapeutic agents, thus providing new pathways or strategies for disease diagnosis and treatment. Specific chapters discuss protein delivery, drug delivery, tissue regeneration, bioimaging, bio-detection, molecular imaging, nucleic acid therapeutics, and DNA-based nanomaterials.

Furthermore, the book focuses on a unique subset of nanomaterials originating from biological entities and explores their potential as nanomedicine tools for selective drug delivery and molecular imaging. Bionanomaterials hold great potential as naturally occurring nanomaterials with enhanced properties, such as biodegradable, biocompatible, safety, and amenability for chemical and genetic manipulation to impart new surface functionalities for the selective targeting. Subsequently, such nanomaterials will enhance the therapeutics payload delivered selectively to the diseased cells. Besides, nanomaterials should have a higher signal-to-noise ratio, making them ideal as molecular imaging tools. Bionanomaterials can be produced in larger quantities at low cost, and most importantly, they are highly monodisperse, making them ideal candidates as tools in nanomedicine. Their composition in terms of chemical and structural point of view is unmatched to their synthetic counterparts, and as they comprise natural amino acids or natural monomers, they are safe and hold no side effects for the development as tools in nanomedicine and drug delivery.

The book series will be an international collaboration to present a comprehensive overview of bionanomaterials from natural sources and explore their benefits, advantages, and disadvantages compared to their synthetic counterparts. It will mainly help in practical academic research innovations in the areas of bionanomaterials. Furthermore, this book will explore the clinical demand for a subset material comprised of natural sources for advanced applications. The book is directed toward researchers, academics, and higher education students working on bionanomaterials in medical, pharmaceutical, environmental applications.

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CHAPTER 1

An Overview of Biomaterial Toxicity and Excretion

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Abstract: Biomaterial is a growing family of materials with specific physicochemical properties. Significant studies have been made to characterize the potential *in vivo* and *in vitro* toxicity of biomaterials. The cytotoxicity may be attributed to variations in the physicochemical properties, target cell types, particle dispersion methods, *etc*. The reported cytotoxicity effects mainly include the impact on the biological system and organ-specific toxicity such as CNS toxicity, lung toxicity, cardiac toxicity, dermal toxicity, gastrointestinal toxicity, *etc*. Despite cellular toxicity, the immunological effects of biomaterials, such as the activation of pulmonary macrophages and associated inflammation, have been extensively studied. In this chapter, the latest research results on the toxicological profiles of nanomaterials, highlighting both the cellular toxicities and the immunological effects, have been incorporated. This analysis also offers details on the overall status, patterns, and research needs for dealing with the toxicological behavior of biomaterials.

Keywords: Biomaterials, Cytotoxicity, Nanocarriers, Toxicity.

INTRODUCTION

With the development of human civilization, biomaterials evolved by incorporating various materials on various lengths from nano- to micro- to macro level with a simple focus on extending human life and improving quality of life. More than 1000 years ago, silver, in various ways, was used as an antimicrobial agent to prevent infection. Different surgical procedures can be found at the very beginning of civilization. However, perhaps the most significant development took place in biomaterials in 1901-2000. Over the past 60 years, the quality of life

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for millions of people has been improved through artificial limbs. Regenerative sutures have simplified surgical procedures, and various cardiac devices have saved lives. The advent of tissue engineering and organ rehabilitation pushes science limits today to make 2001-2100 the most exciting years in the biological field.

The term biomaterial describes something derived from biological sources, and it also describes substances that can be used in the human body as a machine. Polymer science took birth with medicinal polymers in the past, and research continues to expand the performance and stability of these components *in vivo*. Biomaterials are needed in clinical practice as a vital part of permanent implants like large blood vessels, waist implants, catheters, *etc*. In surgery, the early use of polymers has mainly focused on the evolution of connective tissue. Many new systems are emerging due to significant advances in the development and molecular cell biology. The drugs based on lots of unique nucleic acid and protein, which are not administered in the form of pills, provide the impetus for new polymers that can be incorporated to control the delivery of drug and genetic treatment. Tissue engineering has new applications that are integrated with physical requirements where the biomaterials assist the regeneration of body limbs and tissues [1].

Various polymers are utilized in several environmental programs, including polyethylene etherketone (PEK), polysulfone (PS), Silicone (SR), polyethylene terephthalate (PET), polymethylmethacrylate (PMMA), polyacetal (PA), polytetrafluoroethylene (PTFE), polyurethane (PU), polyethylene (PE). The most common composite polymer biomaterials are CF, carbon fiber/ultra-high molecular weight polyethylene (CF/UHMWPE), carbon fiber/epoxy (CF/epoxy), silica/SR, and HA/PE. Polymer materials are used for medicinal applications. Application in various disciplines has been received by polymers, such as tissue engineering, orthopedics, implants, dentistry, ophthalmology, and many other medical fields. Delivery programs designed for polymer enable the slow release of the drug from the body.

An investigation on the use of polymer in genetic therapy has been done. They show safer genetic predisposition as compared to viruses and vectors. Synthetic polymeric materials are widely used for biosensors, experienced devices, and biocontrols. Polymeric essentials should be made biocompatible for biomedical applications. Many of the polymeric systems utilized in the body for medical devices are termed biocompatible, whereas collagen encapsulation after implantation separates them from the body tissues. Polymeric implants may be considered biocompatible if they do not cause adverse responses. When polymers interact with blood cells, a thrombus is generated rapidly. Therefore, items with

blood adhesions that are non-thrombogenic should be utilized for bloodstream contact. Properly balanced polymers employed in treatments should detect and interact harmoniously with living cell components without unspecified interactions. Non-toxic biomaterials are used in various medical and surgical applications. During the growing phase, NDB'S are meant to degrade the body.

Polymers that can be natural or manufactured can be decomposed. The benefits offered by the latter are more significant than the first since they may be flexibly adapted to produce the required property portfolio. Synthetic polymers provide a dependable and immunogenic supply of materials. The standard method includes mechanical characteristics (friction, density, and cutting) and the breakdown time necessary for a given system as a common technique for selecting a polymer to be used as a biomaterial. After completing its goal, it should be lowered to the planting area, leaving non-toxic products. Important issues to consider here are additional biomaterial properties, such as land charge, polarity, distribution of active chemical groups, hydrophobicity, and hydrophilicity (or wettability). It is important to blend polymers with hydrolytically unstable reinforcement for biological control. Esters, anhydrides, orthoesters, and amides are the most active chemical groups [2].

Any synthetic or natural compound except drug, which treats, enhances, or alters muscle or organ function, is called a biomaterial. The most challenging issue to deal with is biomaterial selection due to its biological compatibility and needs. In recent years, material designers have shown much interest. The discovery of different polymers has significantly influenced the growth and control technologies in the tissue engineering industry. However, operators must guarantee that polymer-based biomaterials have long-term strength and dependability to be effective. Utilizing biomedical composite polymer materials gives numerous new choices and design options. Composites composed of polymers can gain several mechanical and biological characteristics that significantly enhance numerous biological applications.

Nevertheless, the manufacturing and marketing of partial or complete medical instruments built from compounds have begun in relatively few situations. Biocompatibility is an essential condition that all living things must meet. The medical study investigates new scientific obstacles to cell/genetic diagnosis, treatment, and prevention. Destroyed biomaterials change over time as the biomaterials undergo mass and surface degradation, leading to changes in the material's surrounding area. Non-corrosive materials also experience changes in chemical and structural properties. However, these changes are not so significant, and the time involved in the physical and chemical changes is much longer than in organic matter [3].

CHARACTERISTICS OF BIOMATERIALS

Biomaterials exhibit some physicochemical properties that must be according to their application. Mechanical properties, corrosion/ degradation stability, *etc*., are the essential physicochemical features of a material. The structure and composition of the material are directly connected to such qualities. It appears evident that the shape and size of biomaterials are of immense significance. For instance, the hip prosthesis's parts have a definite size and shape per the local implant environment. The fate of cell adhesion and proliferation are affected by the mechanical properties of biomaterial such as fatigue strength, young modulus, tensile yield stress, and toughness of titanium. The mechanical characteristics of the implanted material must be very near to that of the substance it replaces (*i.e*., tissue). For example, bone implants like ceramic (bioglass) have higher Young modulus and lower toughness than bone and cannot substitute for femoral or tibial bones [4].

Biological fluids possess dissolved solutes, oxygen, and other oxidants, which cause metals' oxidation rate. Metals like iron that carry a corrosive nature must be omitted due to toxic cations and noble metals exceptions. However, if metal (*e.g*., titanium) forms an impervious and strongly adhesive oxide layer, it will remain stable in the presence of oxidants. When the biomaterial begins to deteriorate, changes occur in the material's structure, which modifies its characteristics. Surface function groups alter the response of biomaterial because the functionality of the surface chemical impacts adsorbed protein and cell/protein interactions [5].

Biomaterials used as bulk materials are intrinsically non-porous, vital to corrosion protection. The pore size, controlled porosity, and connectivity between the pores are significant for bone tissue reconstruction applications. Substitute material such as pure titanium or its alloys are most preferred due to their osteointegration property, but it possesses high elastic modulus than bone. Therefore, elastomeric composites and powdered bioceramics have been developed because they have good mechanical and chemical compatibility with bone. Moreover, biomaterials pore size should be in the range of 400-1000 μm for bone regeneration. Due to the interaction of biomaterial with bodily fluids, physicochemical characteristics of biomaterial must be studied. These interactions take place *via* a surface-tension interface.

Additionally, the contact angle is used to measure biomaterial wettability, which affects biological responses such as blood coagulation, protein adsorption, cellular and bacterial adhesion. Implant materials having hydrophilic surfaces exhibit good biocompatibility and interactions with cells. However, hydrophilic surfaces are less protein adsorbent than hydrophobic surfaces [6].

The behavior of many cell types relies on material surface topography and the rigidity of the underlying material. Ion implantation, polishing, anodization techniques are used to regulate surface topography. Studies showed that human osteoblasts with varying surface roughness adhere to the orthopedic metal substrates (Ti_6Al_4V alloy) [7].

Although the antimicrobial property is not a physicochemical property, it results from a physicochemical modification of that material. The topographic alteration, grafting antimicrobials on the surface, chemical modification, and repellent molecules can acquire that property. Biomaterials must-have antimicrobial properties to avoid the formation of a biofilm resulting in material failure. Peri-implants occur due to the invasion of bacteria in the cavity between the dental plant and gingiva. Bone erosion, inflammation, and eventual implant failure lead to this bacterial infection. Therefore, before implantation, all medical instruments are sterilized [4].

Adhesion and cellular behavior are greatly influenced by biomaterial roughness, which further shows its impact on *in vivo* and *in vitro* results. Rough and polished mirror surfaces have contact areas with cells and molecules. The smooth surface biomaterial is preferred when low friction applications like implants of orthopedic joints are desired, and a rough surface is chosen when utilized for tissue-implant integration, such as end bone implants [8].

TYPES OF BIOMATERIALS

Like any other materials, biomaterials can be grouped into four major categories, *i.e*. (i) Composites, (ii) Ceramics, (iii) Polymers, and (iv) Metals. Polymers can be used in various applications and contain the largest category of biomaterials. In drug delivery uses, polymers are also frequently employed. Collagen, sodium alginate, cellulose, or synthetic polymers such as silicone rubbers, poly(vinyl chloride) (PVC), PMMA, and PLGA may be natural polymers. For dental and orthopedic purposes, metals are frequently employed. Titanium, rubber-steel, and cobalt-chromium alloy are the most often utilized metals. Research on metals as biodegradable agents has recently become more widespread than in polymers. Corrosion resistance has always been one of the main requirements for metal biomaterials, as reported worldwide in literature. In this sense, the idea of considering damaged metal for temporary installation required an inevitable break of this paradigm. The first metal used to decay implants was magnesium (Mg), launched in 1938. However, Mg's degrading behavior was not well known at the time, and the explosion of stainless steel caused the metal to be abandoned. As Mg technology advances, Mg has turned the attention of biomaterials into essential elements of decaying implants. Two iron classes have been proposed,

i.e., Mg and iron (Fe)-based alloys. Calcium phosphates (CaP), aluminum (Al_2O_3), and bioglass are the most commonly utilized biomaterials. The vital component of composite biomaterials is the polymer-ceramic composite. Biomaterials show various interactions with tissue, which can be divided into four different types.

Toxicity: Toxic substances cause the death of surrounding tissues.

Bioinert: Non-toxic but ineffective substances cause this type of reaction. The muscular tissue implantation of bioinert content is caused *in vivo*, leading to the implant's final release and failure. Organic coverage with metal implants can prevent tissue leakage.

Bioactive: This reaction is seen when the substance is non-toxic and practical for life. The term biologically active means forming interactions between substances and tissue handling. Bioactive crystals, many types of polymers, and many calcium phosphate ceramics fall into this category.

Possible findings: This type of reaction is seen when a non-toxic substance dissolves *in vivo*, such as calcium sulfate (Paris concrete), tricalcium phosphate, bioactive glass, and PLGA. As a result, surrounding tissue may be replaced by synthetic materials.

APPLICATIONS OF BIOMATERIALS

In therapeutic application, polymers have played significant roles (Fig. **1**). The features of the biomaterials are (i) flexibility, (ii) resistance against biochemical attack, (iii) excellent biocompatibility, (iv) weight lighting, (v) good physical and mechanical properties available in a wide array of compositions, and (vi) readily manufactured in the required form [9].

Tissue Engineering

Biomaterials regenerate wound spaces as a unique natural matrix and in 3D cell supporters. Polymers are the most prevalent tissue engineering organisms due to their flexibility and similarities in the tissue structure [10].

Thermoplastic polyesters like polycaprolactone (PCL), polyglycolic acid (PGA), and polylactic acid (PLA) are being used as biomaterials. Polyester, a class of polymer, contains an active ester group in its primary series. Esters are the product of carboxylic acid-based chemical compounds (COOH) and hydroxyl compounds (OH), generally alcohol. In the human metabolism, several esters, like fatty acids, are discovered and compatible. For simple hydrolysis, several polyester materials may break down natural compounds. While they are well-characterized and engineered to imitate soft bone chemicals, mechanical

coherence between thermoplastic polymer and live tissue is typically lacking. Disposable biomaterials are suitable as tissue-making sprays as they disrupt the development and growth of new tissues, and unwanted long-term reactions are prevented. The destructive biomaterials used in tissue engineering must go hand in hand, and the degradation effects must be disastrous. The time required to complete dependence damage depends on the target application and functional tissue structure [11].

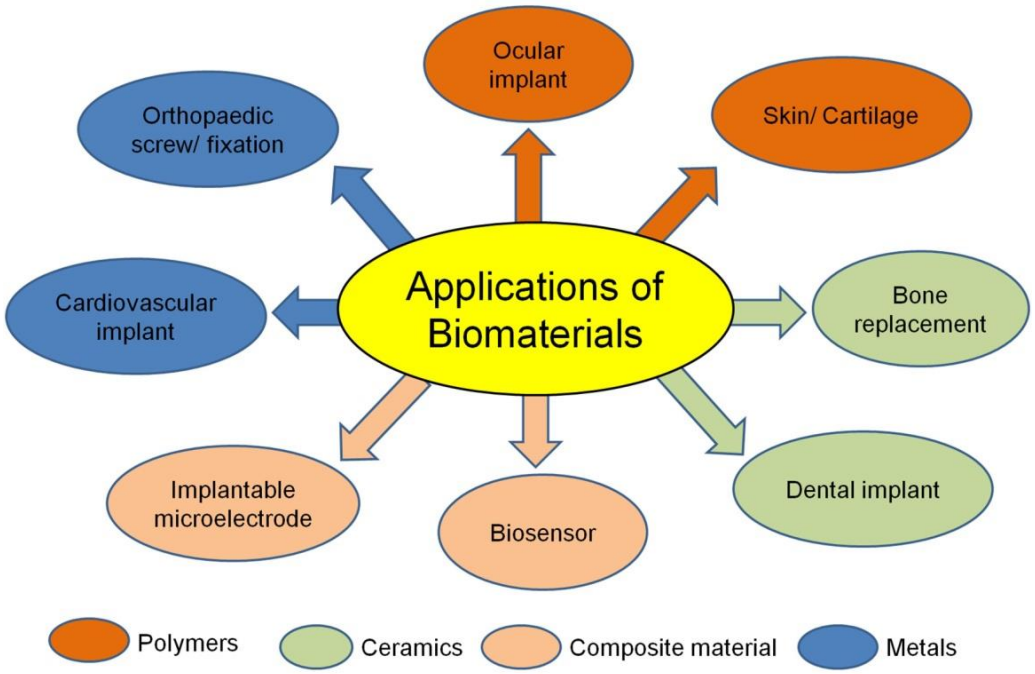

Fig. (1). Different applications of biomaterials.

Implantation of Medical Devices

Perishable synthetic polymers have drawn a great deal of attention to the performance of medical devices. The drug-eluting stents (DES) were frequently utilized in coronary artery disease patients as an automated therapy. Decomposing polymers are commonly used in stents to control the release of drugs as a decaying and recyclable covering. In addition to being used as a decay coating, decomposing polymers are also material for decomposing decaying stents due to their efficient drug-resistant properties and machinery efficiency to prevent stents from cracking [12].

Bone marrow transplants have more advantages than non-corrosive materials or steel. These can relocate the pressure over time to the affected area as it cools,

allows tissue, and does not need a second operation to remove implanted equipment. It is observed that much commercial orthopedic replacement equipment such as nails and crutches for joint repair and screws and maxillofacial repair plates consist of PLLA, polyglycolide, and other perishable polymers. Various devices are frequently constructed from non-corrosive plastic, leading to significant environmental and economic concerns, such as syringes and surgical gloves. PLA, polyglycolide, poly-d, l-(lactide-co-glycolide) (PLGA) can be compromised. Therefore, the adoption of disposable medical equipment that fulfills environmental standards is promising. These decomposing polymers have been utilized to repair and develop some disposable medical devices [13].

Joint Prosthesis

The joints allow our body and limbs to move. The majority of joints inside the body are synovial, allowing freedom of movement to perform various body functions such as walking, running, jogging, jumping, turning, bending, standing, and sitting. Synovial joints are present in the elbow, knee, hip, and shoulder. The most common combination of implants is Total Hip Replacement (THR). The typical THR is an acetabular type cup, and the femur section with the head is incorporated into the acetabular cup, which allows movements. Flexible thickness, high UHMWPE, and plastic distortion are all possibilities. The temporary performance of UHMWPE acetabular cups is acceptable; however, their long-term use is challenging. Geometry and biomechanics of movement in a total knee replacement (TKR) are more complicated than in a hip joint. Knee degeneration and injuries are more common as compared to other joints. The femora and tibia are the significant components of a typical TKR. A metal tray supports a section of the tibia composed of UHMWPE polymer. Although the compound may not be optimal for conveying low concentrations on its own, the combination of composite substrate and UHMWPE is more advantageous. Finger joints, ankle, shoulder, toe, wrist, and elbow are among the other joints that can be replaced. Because of the low success rate of these joint replacements due to the flexibility of the transplant components, they are utilized less frequently than TKR and THR. Silicone rubber (SR) is being investigated for specific sorts (space-filling design) of finger joint replacement. Rupture of the prosthesis and roughening of the arthritic bone is a serious issue. It has been reported that a combination of implants has also been shown to reduce pain, improve stability, increase manual labor, and provide an adequate range of motion [14].

Bone Repair

Collagen fibers with hydroxyapatite nanocrystals pressed alongside collagen fibrils made bones. The lower fibers of the elastic modulus collagen are aligned

with bone and stress indicators. Hydroxyapatite mineral having high elastic modulus contains about 70% of dry bones and enhances bone strength. Internal fixation and external fixation are the two categories of orthopedic treatment. External repairs do not require cracks opening, whereas internal repairs require a fracture site's opening. Plaster bandages or imitation materials are used to create wrists, cones, or metal. Composite material such as spreading material is made of woven cotton cloth, Plaster of Paris, and other reinforcements, including glass cloths and polyester fibers. Cement has been made of glass or polyester, and petroleum products are popular. A good imitation material must have these characteristics- easy to carry and remove, compliant with anatomical shape, lightweight, radiolucent, and waterproof.

Due to the lightweight, the external fixators built of CF/epoxy composite biomaterials are attaining [15]. Bone plate application materials like polyester and polymers like PTFE and PA should be of sufficient strength for fatigue (such as stainless steel), as orthopedic equipment is subject to very high rotating loads and should not be led to heavy rains in a fractured area, which could sway the skeletal union. Non-removable composite plates are composed of thermoplastic composite materials or a thermoset polymer. Non-renewable thermoset compounds include CF/epoxy and GF/epoxy. Researchers have produced CF/PP, CF/PMMA, CF/PEEK, CF/PE, CF/PS, CF/PBT, and CF/nylon thermoplastic, non-resorbable composite bone plates as the technology to create high-quality thermoplastic composites. In several research, CF/PEEK has been reported to be compatible with improved resistance to hydrolysis and radiation (sterilization method) reduction. Other significant characteristics include resistance, high strength, and inertness of organisms with no carcinogenicity or mutagenicity [16].

Drug Delivery Systems

The polymeric material is utilized as a drug delivery device *via* new approaches, including biodegradability in the system. Many perishable polymers - a variety of natural and synthetic materials can be helpful for this purpose. Drug delivery system advancements have emphasized the use of purposely humiliating medicinal polymers. The limitations of conventional drug delivery modalities, like pill or injection, are well understood. A popular approach is to combine drug within the polymer or embed it in the polymer matrix and then implant it to spread into the tissues. In some circumstances, the release process is influenced by polymer erosion or melting. Chitosan is preferred in drug delivery, such as drug conjugate, hydrogel system, breakdown release system, and PEC. Disposable polymers such as poly orthoesters and polylactic acid are employed in drug delivery methods. For several goods, chitosan is preferred in drug delivery, such as drug conjugate, hydrogel system, breakdown release system, and PEC.

Protein/peptide delivery, antibiotics, anti-inflammatory drugs, genetic treatment, growth factors, and bioimaging applications utilize chitosan-based systems. Polyelectrolyte complexes of chitosan particles were produced for the delivery of nasal protein. Insulin-loaded chitosan nanoparticles (NPs) have been proven to increase protein absorption more than suitable chitosan solutions [17].

Heparin is mainly utilized as an antiplatelet and anticoagulant agent. As heparin's routine administration increases bleeding risk, direct site delivery of heparin to damaged vessels offers an excellent substitute to system delivery. The effect of heparin loading on endothelial cell damage is assessed by a mixture of heparin powder and melted polymer. *In vitro* extraction investigations have revealed that loaded heparin may be recovered completely, provided that the heparin stays inert throughout the polymerization. The findings indicate that heparin within polymeric devices for local medication delivery looks promising [18].

TYPES OF TOXICITY

Nanocarrier toxicity can occur in several ways. It can be hazardous since it develops new species by interacting with biological processes. In some cases, NP formation itself causes toxicity. As a result, a comprehensive toxicity investigation is required for any nanocarrier to be considered for *in vivo* treatment. As previously stated, the treatment method can influence the toxicity of nanocarriers [19].

When NPs consist of two or more components, then the toxicity of each ingredient should be investigated. However, a recent study proved the synergistic effect of NPs and Polysorbate 20, a surfactant often utilized in NP systems to aid dispersal and performance. Although gold nanoparticles (AuNPs) and individual surfactants exhibit less toxicity in zebrafish, the concentration of toxins has increased significantly. These findings highlight the importance of undertaking toxicological research on the NP of interest and the matrix in which it will be given [20].

Hepatotoxicity

The most common source of NPs is the liver because the NPs will be placed, digested, and disposed of there, which is why liver toxicity is often considered first. The AuNPs are widely investigated for their extensive pre-surgical and clinical applications in various NPs. In the lower concentrations, the AuNPs are safe because of their chemically neutral properties. However, there have been findings of AuNP poisoning, which is often the result of NP size, surface chemistry, and shape. Research was conducted to assess the potential toxicity of AuNPs. Naked AuNPs (8 mg/kg/week dose) with the size range of 3-100 nm were

introduced in BALB/c mice. At sizes 3, 5, 50, and 100 nm, the AuNPs showed no adverse effects; however, AuNPs caused severe illness in mice, ranging from 8 to 37 nm. Pathological studies of large mice in sick groups exhibited an increase in liver Kupffer cells, increased white pulp in the brain, and integrity loss in the lungs. These toxic symptoms were because of the presence of AuNPs in infected areas. Despite the improvements in cytocompatibility provided by biological coverage, liver toxicity's primary mechanism is thought to result from a significant change in inflammation and subsequent apoptosis [21].

Increased production of reactive oxygen species (ROS) causes the toxic effects of iron oxide NPs. This produced ROS, continuously releasing lipid peroxidation and DNA damage. More efforts are needed to develop a magnetic iron oxide NP with more advanced engineering in the field to detect less toxic, diverse applications in biomedicine. A study has reported that excess iron due to high doses of iron oxide NPs may be detrimental to cirrhosis due to the manifested effects of disruption of iron homeostasis, elevated lipid metabolism, and possible loss of liver function [22].

The toxicity of the metal itself poses further problems with NPs containing heavy metals. Under certain conditions, a study found that cadmium selenide (CdSe)-cored quantum dots (QDs) were very hazardous. The CdQDs cytotoxicity was determined by analyzing parameters during fusion, ultraviolet light exposure, and ground coverage. Data also suggested that cytotoxicity was associated with the release of Cd-free ions. Properly coated CdSe-core QDs are non-toxic and employed for *in vitro* reorganization and tracking of cell migration. In a study, the cytotoxicity of poly(ethylene glycol) (PEG), -NH_2, and -COOH functionalized CdTe CdSe cored QDs with zinc sulfide (ZnS) shell was examined. The cytotoxicity of each type of exposure was assessed by monitoring macrophage cell activity and performing a double hydroxide extraction. After 48 hours, all QDs tested were found to produce considerable cytotoxicity, and coating materials and Cd ions emitted were suspected to be the causes. The deterioration of the materials apparent smoothness led to the release of ions and subsequent toxicity [23].

Nephrotoxicity

Nanoparticles show potent nephrotoxicity at both glomerular and tubular levels as per *in vivo* studies. With NPs sizes less than 6 nm, the kidneys have been studied as a target component of NPs. Nephrotoxicity is noticed, for instance, when CdSNPs are regulated by gavage. One proposed explanation is that Cd can generate ROS and bind cysteine residues to proteins. Iron oxide NPs were tested with a base size of 10 or 30 nm and applied PEI (SEI-10) or PEG (SMG-10,

SMG-30) to detect variations in cell acquisition, clearance, biodistribution, and toxicity. *In vitro*, SEI-10 demonstrated intense cell proliferation in cancer cells and macrophages, showing early toxicity. Iron oxide NPs were introduced parenterally into SKOV-3 tumor-bearing mice.

The SEI-10 (negatively charged) has a high kidney transplantation rate, but PEG-iron oxide NPs were mostly eliminated from the spleen and liver. The SEI-10 injected rats had a 1.5 mg/kg high tolerance dose. However, mice injected with SMG-30 or SMG-10 at doses up to 5 mg/kg had no fatalities [24]. The renal system removes NPs. The modified QDs were covered with a neutral or zwitterionic coating that inhibited the release of serum proteins, kept their modest size (6 nm), and were fully cleansed by the kidneys by passing a unique structure of numerous glomeruli cells. A little contact between NPs and kidneys reveals low nephrotoxicity. Those without a protective layer grew more than 15 nm and were kept in the liver and mirror [25]. However, a recent study showed the drawbacks of the relationship between size and kidney. How these NPs will be eliminated is a question that needs further investigation [26].

Cardiac Toxicity

Sensitive organ systems should also be tested with any NP intended for translation. It has been demonstrated that AgNPs showed immediate toxicity on myocardial electrophysiology and cause symptoms of dependence in rats, which involves undiminished resting energy, possible decrease in heart function, loss of happiness, and fatal bradyarrhythmia [27]. These toxic pains did not occur in mice treated with Ag+ ions, demonstrating that the toxins originated in AgNPs without silver exposure.

Brain Toxicity

Nanomaterial systems pose a considerable threat to the central nervous system (CNS) and brain. The blood-brain barrier (BBB) controls the entries of circulating molecules in the bloodstream to the brain. As a result, the crossing of the BBB by drug delivery pathway has become the focused area [28, 29]. There is no specific route for NPs to be delivered in the brain, and they may pass through cerebrospinal fluid, blood, lymph system, and olfactory mucosa. These are some established approaches; however, they show limited efficacy. Continuous research for the application of nanomedicine in the treatment of deadly brain illnesses and attention to minimizing the potential nanotoxicity is going on [29].

Lung Toxicity

Inhalation is one of the most direct and promising routes of drug administration to the pulmonary system. Exposure may not be unintentional, as in identifying or treating NPs, or unintended, by inhalation or dermal exposure due to the increased number of industrial applications of the NPs. Its toxicity relies upon size, composition, and intended use. The therapeutic lung applications of NPs usually focus on the diagnosis and treatment. Most of these applications rely on blood flow and the pulmonary organ system. These NPs are usually eliminated by the MPS system and do not carry poisons directly to the lung. A report considered the size effect and focused on AgNP toxicity. *In vitro*, Ag-15 nm, Ag-30 nm, or Ag-55 nm in the concentration of 10-75 g/mL interacted with alveolar macrophages. Cell morphological and performance alterations, glutathione and cytokine concentration, and ROS were assessed. These showed concentration and size-dependent toxicity after 24 hours. The Ag-15 nm particles were found to be the most destructive to the cells. The cytotoxicity could be caused by increased ROS and cytokines (monocyte chemotactic protein 1).

In a study, 24 NPs having the same size but different shapes were tested for lung toxicity using two lung cell lines, A549 and THP-1. Toxicity was estimated by 3-(4,5-dimethylthiazo(-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and neutral red tests. Titanium, zirconium, cerium, and aluminum NPs were the most hazardous, with TC_{50} values less than 15 g/mL, followed by copper and zinc NPs. Intratracheal instillation of copper oxide NPs treated the C57BL/6 mice lung model. Within seven days of implantation, inflamed cell infiltration and lymphocyte fusion were found, which intensified after that. Granulomatous inflammation, fibrosis of the lungs, an increase in ROS, and epithelial cell death were also seen [30].

MECHANISM OF TOXICITY

Toxic substances can cause biomembrane damage, impairment of metabolic functions, and genotoxicity in cells. Membrane damage occurs when a hazardous substance interferes with creating a biological membrane by increasing penetration and modifying water transport, ion transport, and metabolite transport mechanisms. These events can eventually lead to cell death. Surfactant-like chemicals, residual monomers, and plasticizers are all components of potentially harmful biomaterials. Active substances like residual monomers, heavy metal ions, or radicals can damage cell metabolic function by inhibiting the activity of the cellular enzyme. The harmful effects of these compounds can cause a decline in energy generation and synthesis of protein, resulting in the alteration of active transport through cells, decreasing cell proliferation, and ultimately cell death.

The generation of DNA damage by binding a chemical; covalent to DNA, affecting the structure and quantity of chromosomes and genes, is genotoxicity. Many studies predict that an organism's genotoxicity uses a variety of bacteria or mammalian cells. Indeed, it should be underlined that most genotoxic chemicals cause gene damage. A highly high concentration causes nuclear disintegration, which allows the cytotoxic impact of a specific chemical to be determined. Various ceramic substances (such as aluminum nitride, sodium titanium dibromide) and monomers (such as methacrylate) exhibited cell toxicity due to direct or indirect interaction between cells and the test material. Direct interaction involves the transplantation of cells where there is a test material. The restricted growth of cells adjacent to that material indicates a toxic effect. Internal toxicity was discovered when fibroblasts were cultured on several biomaterials that did not produce hazardous chemicals.

Cell Death

After an indirect or direct interaction with biomaterials, cell death is one of the essential elements in toxicology. Cell death assays depend on the cell membrane integrity, which signals the entry of foreign molecules (*e.g.*, neutral redness), or on the loss of other cell molecules (*e.g.*, trypan blue). It was one of the first attempts to analyze cell function when it came to biomaterials. The release of intracellular components into the cell formation site can also identify cell lysis. Hemolysis is another approach based on this idea—the increasing hemoglobin concentration in the supernatant damages the membranes of red blood cells (RBCs). Therefore, hemolysis measurements are considered to indicate biological toxicity. Other chemical components in the suspension site, such as lactate dehydrogenase (LDH), signal damage to cell membranes and are examined in cell cultures with building materials or equipment. The amount of LDH extracted can be measured using a commercial test kit. While the loss of membrane integrity might be thought of as the last option in cell survival, a decline in metabolic performance could precursor cell death. The MTT test is one of the most used cytotoxicity tests. It is based on the deception of yellow tetrazolium (MTT) salts in a purple substance produced by mitochondrial dehydrogenase action. As a result, this change occurs only in living cells.

MTT testing has been employed in various toxicity testing of polymers, metals, and clay. A retrospective study of MTT testing revealed that it could be developed with a randomized controlled trial. The differences in MTT test findings are related to cell counts and mitochondrial dehydrogenase functioning. Because formazan crystals do not dissolve in water, preventing direct spectrophotometric detection, the MTT experiment requires using organic solvents like DMSO or isopropanol. Therefore, a new desalination solution of tetrazolium (XTT) was

developed to benefit significantly. The XTT testing can be a standard test for biomaterials toxicity testing. However, it should be highlighted that these approaches measure the value of various cells, reflecting both inactive and active cells. To avoid misinterpretation, a plasma integrity test, like LDH extract or neutral red identification, may be utilized to supplement these findings.

Apoptosis or planned cell death is characterized by nuclear depletion, decreased cell volume, and increased cell density. The ultimate stage of apoptosis in many cell types is endonucleases reducing nuclear DNA into fragments comprised of 180-base multimers. Apoptosis is known to occur before cell proliferation, as evidenced by the release of LDH and a decrease in cell function, which the MTT experiment may detect. Therefore, specific fragments of DNA-related apoptosis may be the first sign of cell injury and death. Compared with the release of LDH, the efficacy of DNA classification for toxicity testing has recently been demonstrated. Apoptosis diagnostic kits based on DNA fragmentation are available in the market. The DNA sequencing tests are also used for evaluating the cytotoxicity of biomaterial.

The Decline in Cell Adhesion

If the original cell attachment is identified first, reduced cell proliferation can be utilized to determine toxicity—cell adhesion to existing cell monolayers following interaction with biomaterial measures cell death. A suitable amount of time must be allowed for cell growth and proliferation after being exposed to the release substance for testing. After cell trypsinization, the number of adherent cells is determined using microscopy or particle counters. Another way to measure cell adhesion with a visible output is to measure the plating's efficiency.

Alteration in Cellular Morphology

According to research into the morphological changes that occur during cell attachment to compatible and incompatible materials, human fibroblasts are more broadly distributed in the glass. In contrast, cell proliferation in hydrophobic biomaterials is inhibited. Besides this, accumulation, fragmentation, and reduction of cell proliferation were observed. This biomaterial incompatibility is called internal toxicity. Cell accumulation and other morphological changes often precede cellular function loss with cells from the substrate. An agar overlay procedure, phase-difference microscopy, or reddish-red detection can be used to evaluate changes in cell morphology. Immunofluorescence microscopy can be used to identify specific cell structures, such as actin cytoskeleton, and detect morphological changes in cells. Another feature of cellular mutation is the continuous cytoplasm removal, which often involves developing autophagosomes. The cytoplasmic release is a reliable indication of toxicity. However, all

morphological investigations necessitate a well-versed investigator and are subject to individual interpretation. Furthermore, computer image analysis and toxicological investigations have been used to measure cell/morphology status.

Cell Proliferation

Cell proliferation is an essential factor in cytotoxicity testing. The cytotoxic effect of biomaterials will risk cell function by compromising structural or physical integrity (resulting in cell death) and altering reproductive integrity (preventing cell proliferation). Cell proliferation can be calculated in various ways. Cell culture in polystyrene tissue without test/extraction material can be used. Because the nature of the substance contributes to cell attachment and growth, transplanting cells directly into the object to be examined is, in most situations, inaccurate. This can result in a misunderstanding of the material's toxicity in question. The most straightforward measure of cell proliferation is the calculation of cultural cells, expressed in the release of experimental material at different times compared to control.

In most cases, this procedure entails trypsinization of cell culture and subsequent microscopy or electronic counting of individual cells. Another often used parameter is protein content, which is used to quantify cell growth. Chemical processes limit the amount of cell protein. Therefore its number can influence its amount. This limitation of the cell protein content has proven to be one of the most valuable biomaterial toxicity tests; both MTT and XTT testing are effective. Both tests have the limitation of distinguishing between the number of cells and the quantity of mitochondrial dehydrogenase activity. Cell proliferation is recommended to position the increasing labels in the cell cycle's S-section. Thymidine can label cells, and tagged cells can be detected *via* autoradiography or other approaches. The 5-Bromo-2'-deoxyuridine is now utilized to evaluate cell proliferation instead of radioactive isotopes. Monoclonal antibodies are used to obtain a bromodeoxyuridine label that is introduced to genetic material and then incorporated into cellular DNA to determine DNA synthesis. This labeling approach has recently been proven to help in the toxicity testing of solid biomaterials [31].

FACTORS RESPONSIBLE FOR BIOMATERIAL TOXICITY

The physicochemical factors of nanomaterials are crucial (Fig. **2**). There are many non-toxic substances in bulk, but if the size decreases, it indicates high toxicity. In addition, chemical composition, its properties, as well as surface also influence toxicity.

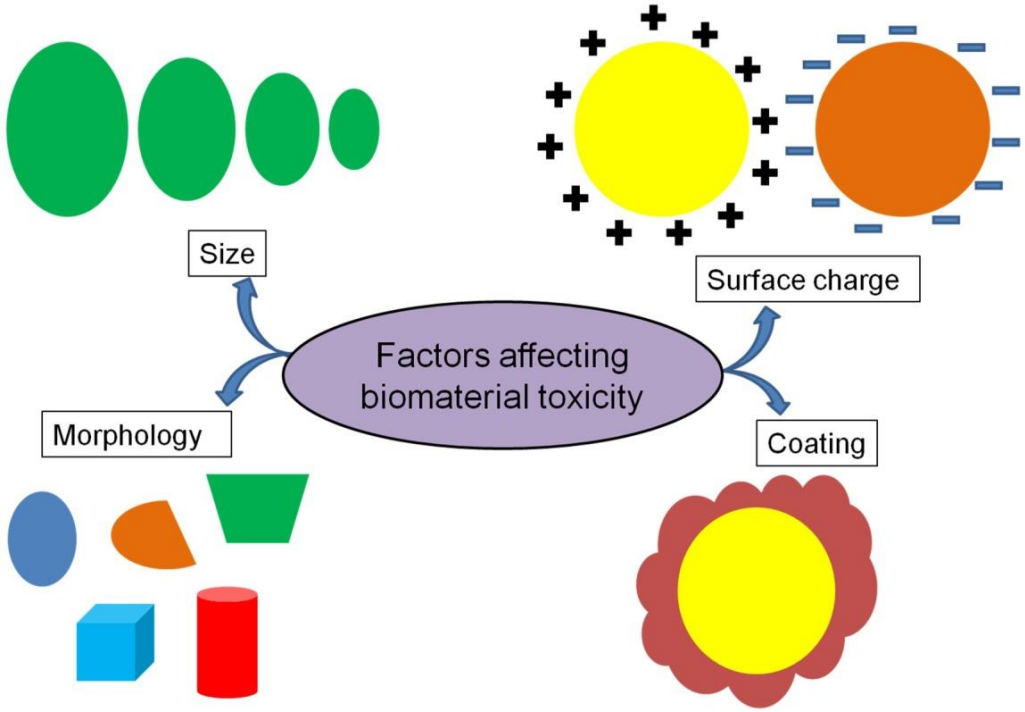

Fig. (2). Factors influencing cytotoxicity of biomaterials.

Particle Size

Particle size is an important parameter determining substance toxicity. The materials cause toxicity when subjected to the cellular environment or absorbed through various routes (inhalation, skin absorption, *etc*.) [32]. As these materials are similar in size to biomacromolecules, they are easily absorbed. The surface area and size of particles are inversely proportional; as the size of the materials reduces, the surface area increases, offering more significant interaction with cellular components.

Several studies have demonstrated the role of particle size in toxicity and confirmed that reduced size is the primary source of toxicity initiation. When NPs enter the cellular environment, they form an NP protein complex that binds with proteins in the cytosolic fluid and causes physiological changes within the cell. Furthermore, the smaller size enhances the formation of ROS. Because the NP size has shrunk, the surface area has grown, resulting in more ROS production sites. The influence of particle size of Ag was studied on RAW264.7 (mouse peritoneal macrophage) cell line and L929 fibroblasts. Genotoxicity, inflammation, developmental toxicity, and cytotoxicity were observed in the AgNPs. The highest toxicity was shown by the smallest AgNPs (20 nm)

compared to larger AgNPs. Ag ions were more toxic to macrophage cells but not in fibroblasts. Effects of titania particles on average human bronchial epithelial cells (BEAS), SmulowGlickman (SG) gingival epithelial cells, lung fibroblasts (WI-38), and oral mucosa fibroblasts (OMFs) were investigated. The researchers wanted to see a link between shape, size, cell lines, and cytotoxicity. The NPs have quickly absorbed the cells; however, they remained aggregated in the cytoplasm.

In comparison to massive rod-shaped particles, spherical NPs were easily swallowed. Depending on the cell type, the toxicity of the NPs differed greatly. The impact of surface functionalization and titania NP size on internalization and cytotoxicity was investigated in this study [33].

The researchers discovered that the cells quickly ingested the NPs smaller than 100 nm. The internalized NPs were enveloped in minute cytoplasmic vesicles. The presence of N+ on the phospholipid layer optimized the interaction of functionalized titania NPs. Even a tiny amount of NPs, when combined with leached ion interactions, damages the cell membrane and contributes to bactericidal activity [34].

The oligodynamic effect of amine-functionalized AgNPs on the cross-linked 3-(--aminoethyl aminopropyl) trimethoxy silane and immobilized silica glass surface was investigated. After 2 hours, cell development on the glass substrate ceased. On the other hand, the utilization of genetic modification in bacterial protein structures as a metal NP resistance mechanism was discussed. These metal NPs make bacterial proliferation difficult while also building resistance mechanisms to metal NPs. It has rekindled interest in developing a new understanding. In QDs, the preparation method influences its size and causes nanomaterial cytotoxicity [35].

QDs are generated by constructing a synthetic channel of an organic ligand like trioctylphospine. These naturally hydrophobic ligands must be treated to become hydrophilic, so the hydrodynamic radius of QDs increases. As a result, changes in nanomaterial size can impact cellular function that can be utilized for therapeutic purposes like drug administration, imaging, and *in vivo* treatment of cancer [36].

Morphology

Particle morphology has a profound effect on cellular function. Various hazardous studies discuss the role of particle size and factor size. Nanomaterials come in various morphologies, including spheres, filaments, planar surfaces, tubes, and so on. Particle structure plays a significant role in reducing toxins only during insertion into the cellular surface. Endocytosis of nanomaterials depends on size

and concentration. During extraction, needle-shaped particles were observed to damage cell membranes [37].

Several other investigations have indicated that size affects mammalian cells. It was discovered that the arrangement of gold morphology in AuNPs improved cellular detection by up to 500% (from rod-shaped to circular shapes) [38]. Several silica NP toxins were investigated in a 48-hour investigation utilizing human neuroblastoma cells (SK-N-SH) at up to 800 µg/mL doses. Single mesoporous NPs were the most hazardous, with relatively little found in circular NPs. The surface area and active groups on mesoporous silica NPs were the primary sources of increased toxicity. Drug delivery efficacy and the hazardous profile (HDF) of silica NPs of various sizes were examined in two cell lines *i.e.*, human keratinocytes (K17) and dermal fibroblasts. The observed toxicity was primarily dictated by the mode of action and cell type. Phagocytosis was a genetic model established inside K17 that involved caveolae-mediated HDF endocytosis [39]. Particles of small size (<20 nm) showed toxic effects due to the morphology of NPs. Two distinct cell lines (fibroblasts L929 and macrophage RAW264) were used to treat nanomaterials for 24 hours. Studies have shown that NPs are naturally cytotoxic and genotoxic, whereas nanowires do not indicate toxicity. The NPs penetrated nuclei and vesicles, causing nuclear damage, whereas nanowires were solely stored on cell surfaces. Toxicity levels have risen due to the increased surface area [39].

Similarly, the effect of compound magnitude on the hazardous effect of nanowires and nanorods was investigated by interacting human leukemia cell lines (THP-1) for 24 hours and then assessing the effect with LDH. Short nanorods were non-toxic, whereas nanorods with intermediate factor ratios did not cause apparent cell death but produced IL-1. Sticks with a high number of characteristics had a high level of toxicity and emitted much IL-1. A recent study investigated the effects of gold nanourchins (NPs with unique morphology) on glioblastoma cells. The PEG-activated AuNPs did not affect cell function or shape, whereas the addition of celastrol increased visibility. The CTAB, on the other hand, transforms AuNPs that are harmful to the filamentous actin, microtubules, and nuclear lamina [40].

Surface Charge

The NPs are introduced into the cell *via* the lipid bilayer lining, and the total charge becomes imbalanced. As a result, well-charged or naturally neutral nanoparticles can be easily absorbed and linked to the cell membrane utilizing electrostatic forces, whereas poorly charged particles bind inappropriately. The kinetics of Au internalization was explored in a study, and it was discovered that cationic AuNPs had a five-fold ingestion rate than anionic AuNPs. The endocytic

pathways of these NPs also vary. The cationic AuNPs spread straight throughout the cells [41]. The interaction of NPs damages the cell membrane's integrity and generates pores. In addition to local charges, ligand interactions also cause variability in toxicity [42].

Coating

It is observed that reduced size, surface charge, ligand presence, and shape characteristics contribute to a substance's toxicity, *e.g.*, toxic iron ions dissolved on the cell surface in various ways resulted in a great extent of toxicity. As a result, research has been undertaken to improve the coating around these metal oxides to reduce leaking circumstances. Further, these coatings reduce toxic levels, tighten particles, prevent binding, and simultaneously increase cell availability. Covering potentially dangerous molecules can help various applications, including drug administration, imaging, and cancer treatment [43].

Chemotherapy medications frequently result in the loss of healthy cells and toxicity. As a result, investigations on selective cancer cell targeting employing compounds coated with nanocarriers are being conducted. The investigations found that coating Fe_2O_3 NPs with chitosan reduced cellular damage and ROS generation, affecting the overall toxicity of NPs. A PEG-coated superparamagnetic iron oxide nanoparticles (SPIONS) minimized the harmful effects of NPs by preventing their interaction with ROS produced in the cytosolic environment, giving an antioxidant cell pathway an acceptable approach of lowering ROS [43, 44]. The cytotoxicity of nickel ferrite was investigated concerning particle size and local charge. The uncollected NP demonstrated dose-independent toxicity with no effect of particle size. However, the oleic acid bound by the NPs showed volume-dependent toxicity. The coating made the hydrophobic coating hydrophilic. Hydrophobic coatings are toxic and are the polar opposite of hydrophilic coatings.

The influence of the AuNPs intake on cell capture and cytotoxicity was tested. Poly (acrylic acid) (PAA) and CTAB coat the AuNPs. The CTABs covering on AuNPs showed low cell performance regardless of their size. On the other hand, the PAA-covered AuNPs showed a dynamic attribute. Free CTAB molecules were the leading cause of the toxins without their cost-effectiveness. Another study used rat cell macrophage lines (RAW-264.7) and lung epithelial cells to investigate the effect of polymer coating on the cytotoxicity of different AgNPs. The type of cover showed an essential effect on toxicity. Poly(diallyl dimethylammonium) treated AgNPs exhibited very high toxins levels, while unmixed AgNPs showed very little. In addition, lung cells showed more resilience in AgNPs doses than macrophages. A study estimated the impact of size and two

different types of AgNPs coated with poly(vinylpyrrolidone) (PVP) and citrate on BEAS cells. Cell detection or toxicity showed no significant difference in aggregated AgNPs. However, very small AgNPs (10 nm) demonstrated the highest level of toxicity caused by large amounts of released Ag ions. *In vivo* photoacoustic imaging therapy was performed using NP composite that displayed high photothermal efficiency. MTT assay of 4T1 murine stem cells was used to assess the biocompatibility of NPs [45, 46].

BIOPHARMACEUTICAL PROFILE OF BIOMATERIALS

Absorption

Transfusion of nanocarriers (NCs) in the blood following subcutaneous (s.c.) administration, inhalation, or dermal administration involves two processes, *i.e*., contact with the body's internal environment and passing through the bio-barrier. The NCs are delivered to regional lymph nodes before reaching the systemic circulation. These management measures can offer a gradual release of the medicine for a typical administration. In contrast, i.v. NC administration leads to immediate contact with the blood [47]. This can result in blood capillary occlusion and embolism development in the event of large NPs. Orally administered NCs can enter epithelial barriers directly by non-transcellular or paracellular distribution or indirectly due to cellular carcinoma followed by transcytosis. By inhalation, NCs can cross the lung barrier and be trapped and removed from the micro lung envelope. The NCs applied to the skin must pass through the epidermis and dermis. The stratum corneum (the epidermis superficial layer) impedes absorption. Only small NCs can enter the stratum corneum in general [47].

Distribution

After absorption, NCs are distributed throughout the body and spread to the tissues, accumulating if controlled for extended periods [48]. The size, shape, and surface charge of the NCs can be optimized to increase blood circulation and improve a specific site's orientation. Blood-derived NCs, on the other hand, can contain plasma components that influence their variability and size, as well as their physicochemical features. A mononuclear phagocytic system (MPS) and its subsequent approval from circulation may also be used to analyze the plasma proteins in NC [49]. Although basic guidelines for NC biodistribution have yet to be defined, several elements may influence the process. First, smaller NCs are better distributed in the muscle than their partially larger counterparts because those with a 200 nm size are usually removed from the spleen. Second, NCs with 10-200 nm tend to disperse the tumor tissue at higher concentrations in cancer treatment because of EPR. Third, a non-circular rather than a round shape

increases rotation time and improves concentration. Modification of NC's performance can improve their identification ability [50]. Folic acid is a ligand widely used to target cancer cell membranes and enhance NC endocytosis through the folate receptor, often strongly over-expressed by cancer cells. Anisamide accomplishes the same aim by interacting with sigma receptors on tumor cells showing excessive stress signs. Glycyrrhetinic acid and galactose are commonly employed to increase the distribution of NCs in the liver. Peptides, proteins, nucleic acids, and nucleic biotin can be modified to better DDS control [51].

Metabolism

The metabolism of NCs largely depends on the absorption pathway. NCs can be removed from the body by structural degradation in a non-destructive manner. Some NCs are generally considered resistant to degeneration and body composition and show prolonged stay in the body. PEGylated QDs, for example, can last for at least two years in the body. However, due to the acidic nature of lysosomes, aberrant NC breakdown can create ions, which can bind to many biomolecules [52 - 54]. Furthermore, chemical groups on the surface of specific NCs can be rendered enzymatic or non-enzymatic degradation.

Enzymatic degradation of organic NCs (those made up of organic MDNS) is influenced by their chemical composition and physicochemical features. Organic NCs may first dissociate from MDNS before combining with less critical genes. *In vivo*, PEGs were discovered to be separated from polymeric NCs [55]. MDNS is significantly more prominent in the liver than the transcellular pores that can be filtered between sinusoidal endothelial cells, and the smaller MDNS can break the pores, reach the Disse space, and enter the hepatocytes. Monooxygenases, transferases, esters, and epoxide hydrolase are some of the enzymes that can break down MDNS. The NCs can produce various metabolites of different sizes, shapes, and chemical forms [56].

Excretion

Excretion is one of the most important methods for reducing the potential toxicity of NCs. Glomerular filtration is used to remove renal clearance based on the content, size, and charge of NC. Typically, particles with a diameter of 6 nm can break through the glomeruli, enter the bladder, and be excreted in the urine [57]. However, a surface charge impacts particle release with a 6-8 nm diameter since positively charged particles have easier glomeruli skipping time. Once they have crossed through the glomerular capillaries & entered the Bowman's space, several particles experience tubular re-absorption and are returned to the bloodstream. Unabsorbed particles cross the glomeruli and are excreted in the urine. It has been reported that most NCs based on polymers are extracted from urine. Non-

biodegradable nanocarriers have a diameter of more than 6 nm are excreted primarily through bile. Active transport is used for low molecular weight (MW) chemicals, while transcellular transport is used for chemicals with a large MW. Polystyrene NCs of more than 50 nm size, for example, is predominantly removed from the body as unchanged particles through bile. The process of bile excretion is greatly influenced by charged nanocarriers [58]. It has been reported that numbers of particles re-enter the blood circulation; these particles are excreted through urine when not reabsorbed.

Cationic NPs can easily pass through the glomerular filtration membrane. At the same time, anionic equivalents are slower to pass through because of the nature of the glycocalyx negative charges [59], but recent research showed that highly negative NPs are eliminated faster than weakly negative NPs and possibly rapidly than those of positively charged NPs [60]. This was confirmed by Naeye and coworkers that cyclodextrin-based cationic siRNA polymer could emanate from the porous glomerular endothelial cells, also degraded on the basement glomerular membrane under the influence of various negatively charged particles (mainly Proteoglycans like heparin sulfate) within the kidney; therefore, it was excreted through urine. Several biodegradable nanomaterials can be converted into lower-weight molecules within the body. Further, these are easily excreted by the kidney, leading to metabolites and responsible for kidney damage. Poloxamers and cyclodextrin derivatives may cause renal impairment due to their possible toxicity [61].

Hepatobiliary clearance is the primary pathway to remove NPs that haven't been cleared *via* renal clearance. Such as, polystyrene microspheres of more than 50 nm are eliminated by bile [62]. Hepatocytes can only be reached by NPs that avoid the Kupffer phagocytic cells. In the circulatory system, NPs as small as 150-200 nm can leak *via* the sinusoidal fenestrae of the liver or transverse over the sinusoidal endothelial cells to approach hepatocytes [63]. Hepatocytes have been demonstrated to prefer the up-taking of positively charged NPs over oppositely charged ones [64]. The entire NPs or their decomposition products could be eliminated in bile after being digested by hepatocytes. Renal clearance has been the most efficient way to eliminate NPs because hepatobiliary excretion is typically delayed (varying from several hours to months) [65]. According to Jiang *et al.* in the hepatocyte, the efflux of glutathione results in an elevated amount of both cysteine and glutathione within the liver sinusoids, leading to a change of NP targeting, surface chemistry, clearance, and decrease in decrease the affinity for serum protein [66].

NPs surface alteration has been shown to inhibit macrophages in the liver and spleen from trapping NPs, although its effect on renal clearance is unclear. The

PEG coating has increased NPs deposition in kidneys [67] and renal clearance [68]. Probably improved kidney uptake of PEGylated NPs because of bypass the reticuloendothelial system (RES) and has an extended half-life in the blood, it is likely that PEGylated NPs is get accumulated within the kidney [60]. However, some researchers reported that PEG-alteration does not affect or prevent renal targeting [69].

Similar NPs observed to stay unchanged following hepatobiliary transit comprises the lanthanide upconversion NPs. Liu and coworker studied LS180 tumor-bearing BALB/C nude mice were shown to have intact fecal excretion of 18.5 nm NaGdF4: Yb, Er NPs 3 and 14 days after administration, respectively. Due to insufficient quantitative information for another type of NPs, it is unclear if the fecal excretion of unchanged NPs is a trait shared by all NPs types or a characteristic shared by only specific types of NPs. In most cases, the integrity of NPs after the fecal removal is highly reliant on the ability of degradation. In contrast, degradable NPs eliminated in the form of a concoction of aggregates, ions, small size residues, and metalloprotein complex [70].

CONCLUSION

Many polymers have been proposed in the last decade. However, in most cases, little effort has been taken to develop new features for specific medical uses. The study of impaired biomaterial growth is a relatively recent field, and therefore, the formation and integration of new, degraded biomaterials is currently a significant research challenge. Perishable materials, as opposed to non-perishable resources, must meet stringent standards. In addition to harmful pollutants (like residual monomers, stabilizers, polymerization initiators, emulsifiers, and by-products), the potential toxicity of breakdown products and subsequent metabolites should be taken into account. As a result of this assumption, only a few non-toxic monomeric compounds have been used successfully in biomaterial degradation.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 2

Nano-Biomaterials for Immunotherapy Applications

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Abstract: Because of their nano-size, biological compatibility, and ability to precisely engineer antigens displayed, payloads packaged, and destinations targeted, nano-biomaterials are gaining traction as next-generation therapeutic tools. Oncolytic viruses were the first to be exploited in cancer immunotherapy because these are natural cell killers and, in some cases, highly selective for cancerous cells. Further, oncolytic viruses can be engineered to encode immune-stimulators and therapeutic genes. However, for oncolytic viruses to work, it is essential to develop these as viable viruses with the ability to infect. This raises safety concerns and poses hurdles in regulatory approval. To circumvent this limitation, non-replicating viruses and virus-like particles have been explored for immunotherapeutic applications. The advantage of these is their inability to infect mammals, thereby eliminating bio-safety concerns. Nonetheless, concerns related to toxicity need to be addressed in each case. Several virus-like particle candidates are currently in preclinical development stages and show promise for clinical use *via* intertumoral administration, also referred to as vaccination *in situ*. In cases where *in situ* administration is not possible due to the absence of solid tumours or inaccessibility of the tumour, nano-biomaterials for systemic administration are desired, and extracellular vesicles fit this bill. Exosomes, in particular, can provide controlled abscopal effects – a property desirable for the treatment of metastatic cancer. This chapter discusses the state-of-the-art in the development of nano-biomaterials for immunotherapy. With a plethora of candidates in development and over two hundred clinical trials ongoing worldwide, nanobiomaterials hold great promise as effective cancer immunotherapies with minimal side effects.

Keywords: Adenovirus, AAV, Cancer vaccines, Checkpoint inhibitors, CPMV, Exosomes, Gene therapy, Immune suppression, Immunotherapy, *In-situ* vaccine, Nanoparticle, Oncolytic virus, Tumour micro-environment, Tumour remission, T-VEC, VLP, Virus-like particle.

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INTRODUCTION

Immunotherapy warrants the use of tools that stimulate or boost the natural defenses of a patient's immune system to treat or prevent disease. Traditionally, the term was coined to represent treatments for cancer that relied on boosting cellular immune responses, as with T-cell therapy, or manipulating immune regulation, as with checkpoint inhibitors [1]. However, with the world grappling with a pandemic in 2020 and with the realization that is boosting one's immune responses is an effective way to fight a coronavirus infection [2, 3], immunotherapy is now used in the broadest sense for all immune-boosting treatments for infectious diseases and oncology. A significant advantage of cancer immunotherapy for the treatment of cancer over traditional methods is that it treats primary tumour as well as prevents metastasis and recurrence of tumours with few side effects. However, immunotherapy does not work for all patients and all cancers. Therefore, the success of an immunotherapeutic drug in a clinical trial depends immensely on patient selection. Even so, immunotherapy is most effective when supplemented with chemotherapeutic drugs or radiation [4].

Although a new and upcoming field, recent successes in immunotherapy have been catapulted by the application of nanobiomaterials for the administration of immunotherapy. Nanobiomaterials are often the immunotherapeutic agent themselves or a carrier of one. Nanobiomaterials can also lead to activation of immune responses in the tumour micro-environment in case of solid tumours allowing the destruction of cancer cells and cancer remission [5, 6]. Apart from the obvious advantages of nano-size and biocompatibility, what makes nanobiomaterials ideal for immunotherapy applications is their amenability [7 - 10]. Using the knowledge available on structure at the atomic level, as in the case of viruses, engineering is possible to precisely display antigens on the nanoparticle exterior [11, 12]. In parallel, using molecular biology tools for genome engineering, it is possible to introduce functional genes within the nanoparticles to orchestrate immune responses. Moreover, space available within the nanoparticle can be exploited for packaging therapeutics [13, 14] such as drugs or genes or siRNA for delivery either by *in situ* administration at the tumour site or targeted systemic administration [8]. In both cases, side effects are limited. This is a significant advantage over conventional chemotherapy, where side-effects to healthy dividing cells, such as hair follicles, digestive tract, and blood-forming cells, are common [15].

Since the advent of the field of nanotechnology in 1959 by Richard Feynman [16], nanomaterials have been extensively used in material sciences and biotechnology. This chapter focuses on different classes of nanomaterials with applications in immunotherapy. The term nanobiomaterial is used for any nano-

scale product of biological origin, *i.e*., mother nature's own nanoparticle toolkit. Several non-biological nanomaterials are also being developed as immunotherapies with significant promise. These include synthetic polymeric nanoparticles [17], liposomes [18], antigens [19] and peptides [20]. However, these will not be discussed in this chapter. A pictorial depiction of all the nanoparticles discussed in this chapter is presented in Fig. (**1**).

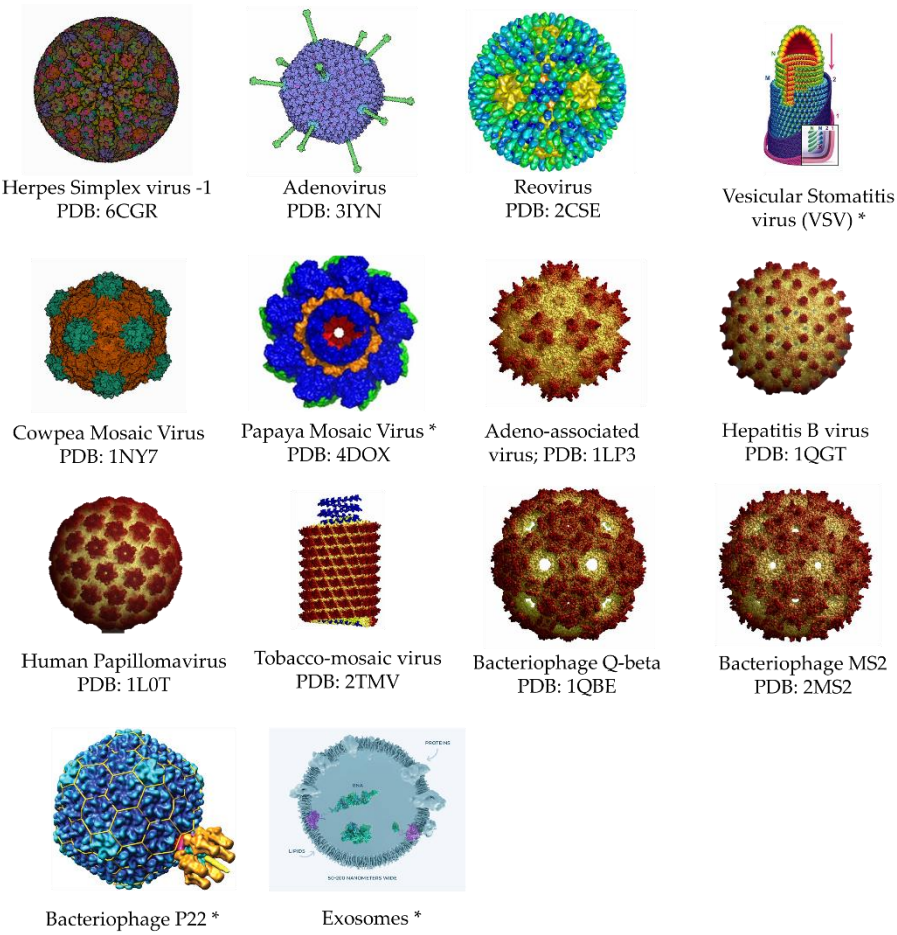

Fig. (1). A pictorial representation of all the nanoparticles discussed in this chapter, in order of their mention. Images are not to scale. Images were acquired from the Protein Data Bank [21] where available and the PDB identification number for each is mentioned in parentheses. Images not obtained from PDB have been marked with an asterisk and duly cited [22 - 25].

IMMUNOTHERAPY & CANCER

This section is intended for those who are not familiar with immunotherapy as it is imperative that immunotherapy concepts are understood to be able to grasp the

rest of the chapter on nanobiomaterials for immunotherapy. The section can be skipped by specialists of the field.

Immunotherapy is best explained using the analogy of a ship: A ship that has very sophisticated machinery and competent staff on board. This ship sails around the world, braving storms, and tides, completing several voyages. However, as time goes on, the ship undergoes routine wear and tear and occasional equipment malfunction. Most of the time, when there is a malfunction, the quality control staff catches it and repairs it right away. However, there can be some, although rare, occasions when malfunctions go undetected. If these are not kept under check or repaired by external intervention, the ship will sink. This ship is an analogy for the human body.

The human body's sophisticated quality control: 'the immune system' is working around the clock to repair malfunctions at the cellular level. The innate immune system consists of macrophages, neutrophils and other anti-microbial compounds that recognize molecules peculiar to frequently encountered pathogens. The adaptive immunity consists of lymphocytes and antibodies, both of which have a high degree of specificity. Lymphocytes (B-cells, T-cells and natural killer cells) respond to the foreign challenge, such as pathogens, and memory is induced after a single response to one specific pathogen. Together, the innate and adaptive immune systems fight off foreign invaders such as viruses and bacteria [26].

A lesser-known fact is that the immune system also conducts surveillance and protects against cellular malfunctions on a regular basis. In a rare genetic event, one's own cells evolve to not be conducive to immune surveillance resulting in immune suppression. That, is the start of the dreaded disease called cancer [27]. What makes treatment of cancer difficult is the micro-environment of the cancerous cells or tumours, which shuts down immune cells from acting at that site. Therefore, any therapeutic agent must be able to reverse immune suppression in the tumour microenvironment. This type of anti-tumour immune response is also referred to as making a 'cold' tumour 'hot' [28].

As for the ship, once a malfunction is detected, external help may be called in addition to the internal quality control systems. Chemotherapy, radiotherapy, and surgery are external treatments for cancer, while immunotherapy works by strengthening the body's own immune system. A combination of the two (external and internal treatments) is the best regimen for battling cancer.

ONCOLYTIC VIRUSES FOR IMMUNOTHERAPY

Immunotherapy exploits the natural ability of viruses to kill host cells to fight cancers such as melanoma. Apart from direct cell killing, oncolytic viruses can be

engineered to stimulate innate and adaptive tumour-directed immune responses, which result in long-term tumour remission [29].

The first oncolytic virus-based therapy to be approved by the U.S. Food and Drug Administration (FDA) is sold under the commercial name of Imlygic ® and comprises the talimogene laherparepvec drug, abbreviated as, T-VEC [30]. T-VEC consists of a genetically modified version of the Herpes Simplex Virus type 1 (HSV-1] and is administered *in situ* into cutaneous, subcutaneous, and nodal lesions in melanoma patients. First, the virus infects and kills tumour cells, which leads to the release of tumor-derived antigens. These antigens signal to the body's immune system and promote an anti-tumour response. Second, in T-VEC, the virus has been engineered to encode granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is expressed during viral replication and further promotes an anti-tumour response [31]. Additionally, genetic modification of the virus in T-VEC significantly reduces its ability to cause herpes, eliminating safety concerns. The above factors make T-VEC effective for treatment of melanoma.

Despite its efficiency, the FDA approval for Imlygic came only recently, in 2015, and it remains the only approved oncolytic viral therapy today. Unlike traditional drugs defined with their pharmacokinetic and pharmacodynamic profiles, oncolytic viruses rely on their interactions with tumour cells, tumour microenvironment and population dynamics, which makes the process of clinical approval atypical. Ongoing research focuses on combinations of Imlygic with other immune-regulators, such as MEK inhibitors and the PD-1 checkpoint blockade, for tumour eradication and improved survival. The combination therapeutic will have a significant edge over other standards of care for melanoma [32].

In addition to the killing ability of viruses, the specificity of targeting is critical for an efficacious oncolytic viral immunotherapy. Adenoviruses can be engineered for tumor specificity [33] and therefore these have been widely developed for cancer therapy, for liver, prostate and bladder cancer. Engineering for selectivity in virus replication is done by using cancer-specific promoters or microRNAs to regulate adenoviral E1A gene expression [34, 35]. Therapeutic potential has been demonstrated in mouse models for liver cancer without causing hepatotoxicity [36] and *in vitro* using human bladder transitional cell carcinoma cell lines [37]. Oncolytic adenoviruses are engineered to integrate functional genes that induce immune stimulation, either by T-cell priming, antigen presentation or interference with immune suppression. However, it is needed to further reduce the pathogenicity of replicating adenoviruses to make this technology acceptable for clinical usage [38].

Reovirus-based oncolytic therapy or reoviral therapy was developed due to the natural cell-killing ability of reoviruses and their selectivity for cancer cells by exploiting the abnormal expression of Ras in cancer cells [39, 40]. Almost a decade ago, clinical trails of a reoviral product named Reolysin® were conducted by Oncolytics Biotech Inc [41]. in patients with multiple myeloma, *via* intravenous administration. Reolysin showed safety and selectivity, but, failed in terms of efficacy, suggesting some tweaking is required [42]. More recently, combination treatments with reolysin are being tested in cancer cell lines. In combination with DNA-damaging agents such as topoisomerase inhibitors, reovirus has shown to selectively infect and kill triple-negative breast cancer (TNBC) cells [43]. Further, by crosslinking doxorubicin to reoviruses, the reo-dox combination was found to significantly reduce primary and metastatic tumour burden in TNBC cells [44], renewing promise in the suitability of reovirus as an oncolytic virus.

Another oncolytic virus with inherent selectivity is the vesicular stomatitis virus (VSV), because, impaired type I interferon pathways in cancer cells makes cells more susceptible to VSV [45]. Preclinical studies on VSV have shown promise; VSV encoding the interferon-beta transgene showed oncolytic potency in syngeneic murine models of non-small cell lung cancer [46]. VSV is currently undergoing clinical trails for skin, lung, liver and, uterine cancer, under the name of Voyager-V1 by Vyriad Inc., in combination with checkpoint inhibitors [47].

NON-REPLICATING VIRUSES FOR IMMUNOTHERAPY

Given the safety concerns with replicating viruses, in recent years, research has focused on ablation of viral infectivity by deploying viruses that are not capable of replication in mammals, or those with replication inactivated, chemically, or by irradiation. This takes away the cell-killing activity of the virus, while providing the machinery needed to develop an immune response at the tumour site. In addition to natural signatures on the virus that allow induction of local inflammation, engineering is done to display tumour antigens (Fig. **2**), which can significantly boost systemic immune responses. Once cancer-specific antigens are presented to the immune system, dendritic cells take up this antigen and travel to lymph nodes, which are sites of T-cell priming. Subsequently, naïve CD4+ and CD8+ T-cells are primed, and memory T-cells are activated, specific to the cancer antigen. These activated T-cells circulate and interact with their cognate ligand at the site of the tumour, thereby evading immune suppression and initiating tumour lysis.

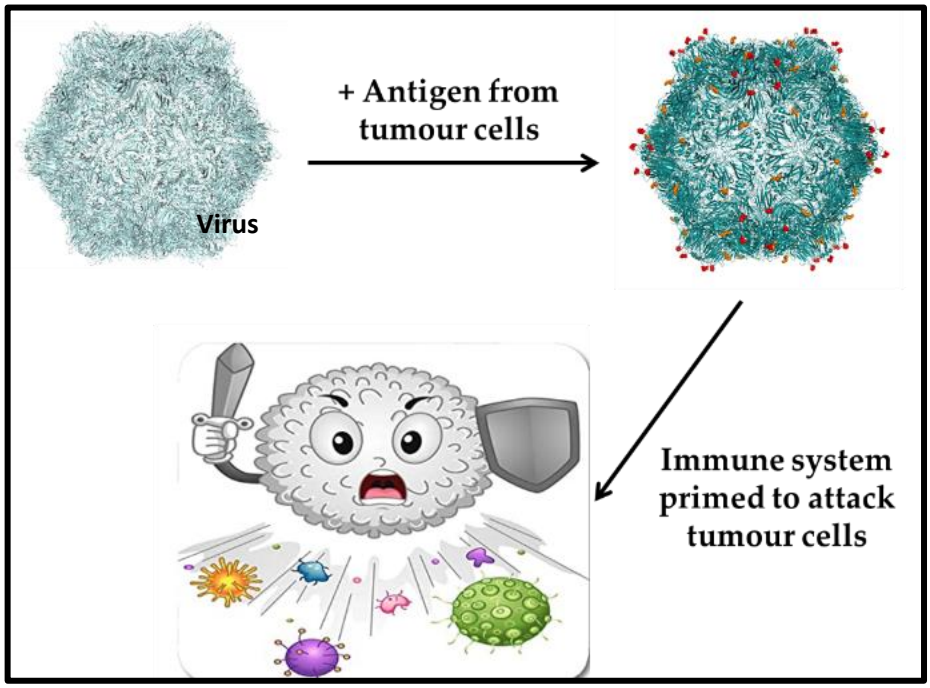

Fig. (2). Antigen display on viral nanoparticles. A schematic depicting the introduction of antigenic peptides (in red and orange) within surface loops of an icosahedral virus (in cyan). Genetic modification of the virus coat protein/proteins results in a symmetric display of the antigen on the nanoparticle platform. Presentation of the antigen to the immune system in this manner results in priming of immune cells to protect against the antigen. If the antigen is specific to a tumour, the nanoparticle vaccine triggers an anti-tumour response. The virus model was generated using Pymol [48] with PDB ID: 1ny7.

A good example of a non-replicating virus being developed for immunotherapy applications is cowpea mosaic virus (CPMV). CPMV is an icosahedral virus of 30 nm and infects legumes such as cowpea plants [49]. Inherently, CPMV is non-infectious to mammals and, further, to alleviate safety concerns, the RNA genome of CPMV can be inactivated using UV treatments [50, 51]. Studies in tumour-bearing mice show that when CPMV is administered directly into a tumour (*in situ* vaccine), strong innate immune responses are raised, and durable anti-tumour systemic immunity is achieved [52]. Comparisons with other viruses of similar structure reveal that CPMV consists of unique features that make it a strong immune stimulant with the potential to activate innate and adaptive immune cells. Additionally, presence of encapsulated RNA in CPMV is believed to result in a distinct molecular signature linked to TLR7/8 recognition [53]. Mosaic Immunoengineering Inc. is a development-stage biotechnology company that is developing a CPMV-based therapeutic candidate named MI-O-101, in order to tap into the potential of this plant virus as an *in situ* immunotherapeutic [54].

Rod-shaped particles of Papaya mosaic virus (PapMV) have also been proposed for cancer immunotherapy, because of their potential to induce to innate immunity *via* toll-like receptor 7 (TLR-7] engagement, using mouse models for melanoma [23, 55]. The case for PapMV is also supported by previous research done on the use of PapMV as an antigen display platform [23].

A subset of cancer immunotherapy is gene therapy, which refers to treatment *via* delivery of a gene encoding an immune-boosting protein, for example, the Interleukin-12 (IL-12) gene that promotes cellular immune responses [56]. Virus-derived nanoparticles are commonly used for delivery of payloads [57] and the single most extensively used virus for gene therapy for cancer is the Adeno-associated virus or AAV [58, 59]. AAV is a single-stranded DNA parvovirus with an inherent ability to deliver DNA to living mammalian cells. The characteristic feature that makes AAV suitable for gene therapy is its deficiency in replication [60]. Recombinant AAV vectors have shown great promise for cancer therapy due to their efficacy, long-term expression, lack of pathogenicity, and, strong safety profile in clinical trials [61]. Using studies on mouse models for glioblastoma and banking on genetic engineering techniques have been developed to make the AAV capsid able to cross the blood-brain barrier, Xu *et al*. propose AAV-based gene therapy as a viable approach for treatment of highly aggressive Grade IV glioblastoma, *via* systemic delivery [62]. The limitations of AAV approaches are in the packaging capacity and efficiency of delivery, and, research continues to improve on these issues [63, 64]. Additionally, replication-defective vectors of retroviruses and lentiviruses are being developed for gene therapy, but with limited success so far [65, 66].

Despite significant developments in the field, approval of viral nanoparticles as cancer vaccines requires alleviation of regulatory concerns over the presence of the viral genome in the vaccine. Virus-like particles, addressed in the next section, circumvent such regulatory concerns.

VIRUS-LIKE PARTICLES FOR IMMUNOTHERAPY

Virus-like particles or VLPs are protein nanoparticles that resemble the native virus in terms of structure and morphology but lack native genetic material (Fig. **3**), rendering them non-infectious and non-replicative. Just like viruses, VLPs have natural immune-stimulating signatures. Additionally, surface loops of virus coat proteins can be engineered to induce targeted immune responses [67]. In fact, a large range of genetic modifications can be applied to the VLP surface, since the VLP does not need to be a viable virus for infection, and, the only property that needs to be retained is self-assembly. Any genetic or chemical modifications developed for a viral nanoparticle can be applied to VLPs.

However, protocols for production, purification, and formulation of VLPs often need to be optimized as the absence of a genome in VLPs negatively impacts the stability of the capsid.

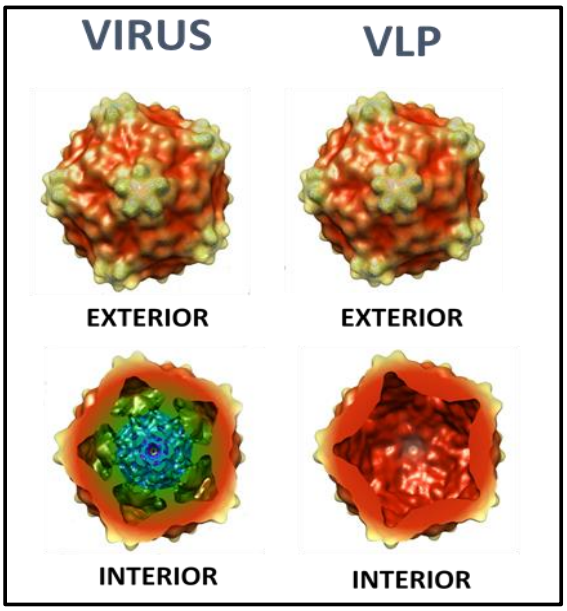

Fig. (3). A virus and a virus-like particle (VLP). In the right panel, the exterior and interior of a VLP is depicted and compared to that of a virus (left panel). On the outside, the VLP is almost identical to the virus, while on the inside, the VLP is devoid of the viral genome. Non-cognate nucleic acid may get encapsulated within the VLP in come cases. Raw images courtesy of Prof. George Lomonossoff, John Innes Centre, Norwich, U.K. and Prof. Neil Ranson, University of Leeds, U.K.

Over the last two decades, VLPs have been developed extensively for uses in nanomedicine [68 - 70]. VLP-based vaccines for two targets, namely Hepatitis-B and Human Papillomavirus, are available globally and have demonstrated safety, high efficacy and durability in adults [71 - 73]. The success of VLP vaccines in treatment of infectious diseases laid the foundation for development of VLP vaccines for immunotherapy.

In the previous section, the potency of CPMV as an *in situ* vaccine for immunotherapy has been described. The observations were explored further using RNA-free particles of CPMV, referred to as CPMV VLPs or eCPMV. It was seen that, for CPMV, the virus and VLP showed a similar profile of immune stimulation, but also that, RNA-containing CPMV induced a higher proportion of potent antigen-presenting cells including activated dendritic cells (DCs) and tumour-infiltrating neutrophils (TINs) [74]. These unique yet overlapping properties indicate the suitability of the virus and the VLP for different

immunotherapeutic strategies. Using a glioma mouse model (GL261), the immunotherapeutic potential of CPMV VLP, as an *in situ* vaccine against intracranial glioma, was assessed. Remarkably, 30 days post tumour inoculation, CPMV VLP-treated mice showed the absence of tumours or regressed intracranial tumours, while untreated mice developed large tumours [75]. Consistent with other studies on CPMV [76], the antitumour response was found to be driven by monocytic DCs and TINs. In addition, reversal of the tumour microenvironment *via* reduction of regulatory T-cells was observed, and, this is thought to be the result of CPMV-induced inflammatory cytokine IL-12. Data, so far, presents CPMV VLPs as a potent, yet safe, localized immunotherapy. From the perspective of translation of this research into clinic, high-level production of CPMV VLPs is possible in *Nicotiana benthamiana* plants [77] and formulations are in development [78, 79]. The risk, however, that is yet to be mitigated is the development of anti-CPMV inflammatory responses in subjects, specifically, cerebral edema in case of intracranial administration. This is especially the case when multiple doses of the vaccine are administered.

Conclusions from work on CPMV have also been also extended to VLPs of another plant virus named Tobacco Mosaic Virus (TMV). TMV differs fundamentally from CPMV in that TMV is a rod-shaped virus with lengths of 50-300 nm (and approximate width of 18 nm). TMV VLPs are generated using a self-assembly protocol *in vitro* where RNA containing fragments from the TMV genome act as templates for assembly of TMV coat proteins. When tested as an *in situ* vaccine in mouse models, TMV was found to lead to recruitment of innate immune cells and induce a potent immune response, characterized by signatures of pro-inflammatory cytokines, such as, IL-6. However, when compared head-t--head with CPMV VLPs, TMV VLPs were found to be less potent, regardless of the length of the TMV rod [80]. This study shows that although CPMV and TMV VLPs both lead to anti-tumour responses and qualify as *in situ* immunotherapeutics, their underlying mechanisms of action are different. While diverse properties of diverse VLPs provide a great toolkit for immunotherapy, a deeper understanding of such differences is imperative for translation to the clinic. Display of cancer antigens on the surface of VLPs has been tested successfully for VLPs of bacteriophage P22 [81], bacteriophage Qbeta [82], Hepatitis B core antigen [83, 84] and bacteriophage MS2 [85]. Proof-of-concept work has been completed in all cases, and, efficacy in animal models has been demonstrated in some, but limited information is available on their mechanisms of action.

Apart from antigen display on the exterior surfaces, VLPs are ideal for packaging of nucleic acids within them. To this end, VLPs of bacteriophage MS2 have been engineered for delivery of RNA. In a proof-of-concept study, the MS2 coat protein was assembled in yeast in presence of mRNA encoding the prostate-

extensive antigen (PAP) and the granulocyte-macrophage colony-stimulating factor (GM-CSF), and, tested in a murine tumour model for prostate cancer. The MS2-based mRNA vaccine was resistant to RNase, non-toxic at the doses tested and efficacious as a therapeutic, demonstrating antigen-specific cytotoxic T-cell responses and a balanced Th1/Th2 response [86] – both desirable properties for a cancer vaccine.

Self-adjuvanting properties, amenability to modifications on the exterior (antigen display) and tolerability to packaging on the inside (payload delivery) makes VLPs lucrative tools for immunotherapy. VLPs have the potential to hit multiple targets *via* varied modes of action. The caution, however, is the development of an immune response against the VLP backbone, especially when the regimen involves multiple doses. Therapy consisting of a combination of VLPs of diverse origin might be the best approach.

EXTRA-CELLULAR VESICLES FOR IMMUNOTHERAPY

Extra-cellular vesicles or EVs are nano-scale lipid shells that are secreted into the plasma by most living cells, including immune cells and cancer cells. The properties of EVs vary based on their origin, and, whether they were secreted under physiological conditions, or, under conditions of stress [87]. Thus, EVs can reveal a lot about the status of their parent cell or tissue. EVs can either be (i) microvesicles that are directly pinched off from plasma membranes, or (ii) exosomes secreted from multivesicular endosomes. In both cases, EVs carry with them specific signalling cues that can influence the tumour microenvironment and distal immune responses. For instance, studies show that tumour cells expressing PD-L1 release exosomes carrying PD-L1, a phenomenon which contributes to overall immune evasion [88]. In layman's terms, EVs are messengers of their parent cells. Interestingly, this attribute is being exploited in development of non-invasive diagnostic methods for monitoring cancer progression [89, 90].

The field of nanomedicine has also found ways to deploy exosomes and exosome mimetics for immunotherapy [91 - 94]. Exosomes displaying cancer antigens or antibody fragments can be administered systemically without concerns of any anti-platform immune responses. Displayed antigens or peptides, once chopped up by antigen presenting cells of the immune system, result in a systemic anti-tumour response, and, reversal of immune suppression in the tumour microenvironment. Displayed antibody fragments lead to immune modulation once they reach the site of the tumour.

In addition to embedding proteins within their lipid bilayer, exosomes have the capacity package proteins inside. Several ongoing studies explore exosomes for delivery of siRNA [95], miRNA [96], chemotherapeutic drugs [97] and anti-inflammatory compounds [98], for treatment of cancer.

CONCLUSION

The 2018 Nobel Prize in Physiology & Medicine recognized the work of James P. Allison and Tasuku Honjo in the field of cancer immunotherapy [99]. Several immunotherapeutic drugs are now in clinical trials worldwide, with rapid bench-to-bedside timelines [100]. Even though the field is recent, advances in clinical trials, and then, the Nobel only go to show how valuable the immunotherapy toolkit is for the field of medicine, and, how many more patients' lives can be saved using immunotherapy for management of cancers, and to some extent, infectious diseases.

Nano-biomaterials are central to these developments in the immunotherapy field, with viruses or virus-like particles (VLPs) at the forefront (Fig. **4**). Combinatorial therapies of VLP vaccines and immune checkpoint modulators are becoming increasingly common in clinical trials. We can expect a surge in virus-based immunotherapies getting approvals for clinical use in the coming years.

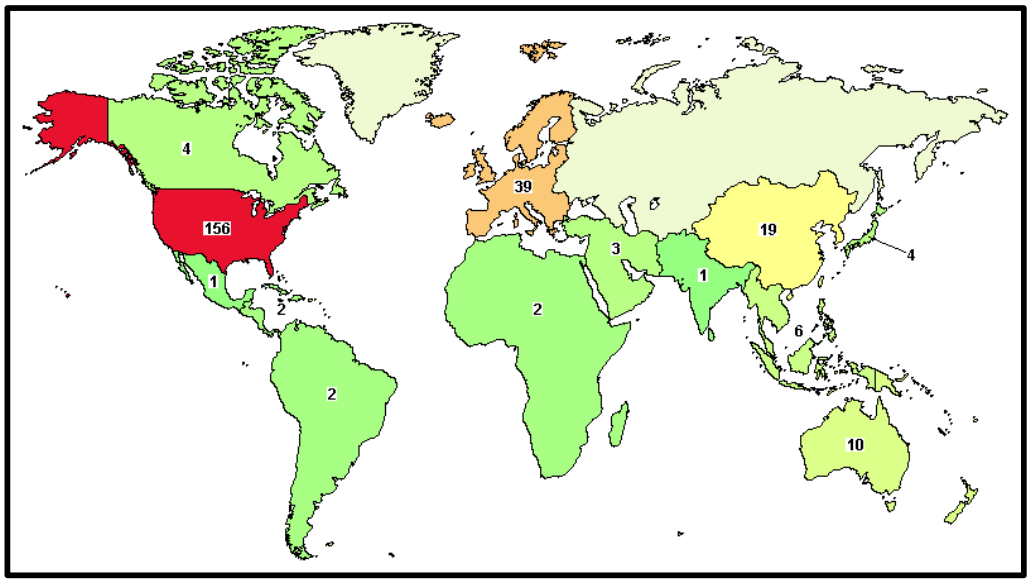

Fig. (4). Number of clinical trials globally testing viral nanoparticles for cancer immunotherapy. A world map with the number of ongoing and completed clinical trials in the field of cancer immunotherapy testing drugs derived from virus-based nanoparticles. Output generated from U. S. National Library of Medicine database [101] for 'all studies' with keywords: 'immunotherapy' and 'virus' for condition: 'cancer'. Accessed March 01, 2021.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest. The author would like to state that the contents shared in this chapter belong solely to the author and have no contribution, in writing or proof-reading, from Medicago R&D Inc. or any such institution. As such, the author claims full responsibility for the views presented.

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CHAPTER 3

Lipid-Based Nanomaterials in Cancer Treatment and Diagnosis

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Abstract: Cancer consists of a wide range of diseases that are mainly driven by the continuous unregulated proliferation of cancer cells. Current treatment options include the use of chemotherapies, radiotherapy, and surgery. Recently, there was an increased interest in applying nanoparticles (NPs) in cancer diagnosis and treatment. NPs are materials in the size range 1 to 100 nm and can be classified based on their properties, shape, or size. They have attracted wide attention because of their versatile physicochemical properties, nanoscale sizes, high surface-to-volume ratios, favourable drug release profiles, and targeting modifications. Nanotechnology can be used to improve the personalisation of cancer diagnosis and treatment by enhancing the detection of cancer-specific biomarkers, imaging of tumours and their metastases, specific drug delivery to target cells, and real-time observation of treatment progression. This chapter will highlight the main types of lipid NPs with their preparation methods. The clinical applications of these lipid NPs in cancer diagnosis and treatment will be presented along with the currently approved drugs based on these NPs.

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CANCER BACKGROUND

Cancer is considered one of the fatal diseases that has reported high incidence and mortality rates globally [1]. For instance, the Global Cancer Observatory (GLOBOCAN) 2020, an online database of global cancer statistics and estimates of occurrence and death rates in 185 countries for 36 types of cancer, has indicated that 19.3 million new cancer patients were diagnosed and around 10 million deaths caused by cancer in 2020 [1]. Therefore, interest in studying cancer continues to progress at a high rate to investigate the underlying causes of cancer and progression.

On a biological level, cancer consists of a wide range of diseases that are mainly driven by the continuous unregulated proliferation of cancer cells [2]. Over growth of cells may develop a mass of tissues called a tumour. However, tumours can be benign or malignant [3]. Benign tumours are usually non-invasive and can be removed without the risk of reoccurrence. Also, cells of benign tumours do not circulate or spread to other parts of the body [4]. At the same time, malignant tumours can invade nearby tissues and spread to other parts of the body in a process known as metastasis [4].

Cancer types are classified into five main categories according to their origin, for instance, carcinoma usually originating from epithelial cells, sarcoma arising from bone, cartilage, fat, muscle, blood vessels, leukemia from the bone marrow, while lymphoma and myeloma are found to be derived from cells of the immune system, and central nervous system cancers from brain tissues and spinal cord [2, 4]. Therefore, different types of cancer exhibit various behaviors and responses to treatment [2].

Cancer initiation and progression are viewed as a multi-step process at a cellular level, where progressive genetic alterations transform normal human cells into highly malignant ones. These genetic changes are found to affect three gene classes: proto-oncogenes, tumour suppressor genes, which are both involved in normal cell growth and division, and DNA repair genes that are responsible for fixing damaged DNA [2, 5]. For instance, any modifications, amplifications, or deletions in these genes may cause a de-coupling of normal cell growth and differentiation [5, 6]. Moreover, these changes in the cell's genetic material may arise unexpectedly or be induced by a factor or carcinogen that causes cancer [2].

Carcinogens include solar ultraviolet radiation, chemicals in tobacco smoke, and viruses. However, carcinogenesis, the process of cancer development, does not

rely only on single causes in most cancers. It has been found that many factors contribute to both animal models and humans [2].

However, preventive measures can be taken against some carcinogens such as radiation and smoking in order to minimise the incidence rate of cancer, as it has been found that more than half of all cancers are preventable [7]. However, integrating effective therapeutic approaches and developing new ones is limited [8].

In recent years, different forms of cancer have been effectively treated by immunotherapies such as antibodies [9], stem cell therapies [10], and chimeric antigen receptor (CAR)-T cell therapies [11, 12]. The success of these approaches is attributed to the high specificity and efficacy of these molecules in treating both primary and metastasised tumours. Despite the high promise of these approaches, a few undesired side effects can also arise, like autoimmune disease [13]. Furthermore, lymphoma and other non-solid tumours have generally shown better responses to immunotherapies than solid tumours [14, 15] due to the expected difficulty in penetrating solid tumours [16]. The immune-suppressive tumour microenvironment can similarly contribute to this efficacy reduction against solid tumours [17]. These limitations can be surpassed through the utilisation of nanotechnology.

NANOPARTICLE APPLICATIONS

Nanoparticles (NPs) are materials in size range 1 to 100 nm and can be classified based on their properties, shape, or size [18]. They have attracted wide attention because of their versatile physicochemical properties, nanoscale sizes, high surface-to-volume ratios, favourable drug release profiles, and targeting modifications [19, 20]. Nanotechnology can be used to improve personalisation of cancer diagnosis and treatment by enhancing the detection of cancer-specific biomarkers, imaging of tumours and their metastases, specific drug delivery to target cells, and real-time observation of treatment progression [21, 22].

The crucial challenge in treating cancer resides in engineering an effective treatment that targets cancer cells without affecting surrounding healthy cells [23]. The NPs must pass through several physiological and biological barriers to be effective. So their use as delivery systems inflicts necessities to optimise their size, surface chemistry, and biocompatibility to avoid non-specific interactions and enable specific binding to their targets. Attia *et al.* [24], specified different criteria that should be maintained by all therapeutic NPs, including the ability to remain stable in the blood and tumour microenvironment (TME); to evade reticuloendothelial system (RES) clearance, and prevent being seized by the mononuclear phagocyte system (MPS); to accumulate in tumour tissues through

irregular tumour vasculature; and to infiltrate into tumour interstitial fluid with high pressure.

Various articles have comprehensively reviewed the use of different nanomaterials as carriers of therapeutic molecules for cancer treatment [23, 25 - 27]. The implemented NPs can be commonly classified into organic (liposomes, micelles, lipids, poly-lactic acid, exosomes, and poly(lactic-co-glycolic acid) [28, 29], inorganic (gold, silver, iron, carbon quantum dots, silica, and graphene), and composites (metal-organic frameworks and transition metals dichalcogenide) [30]. These systems can be additionally extended by introducing external effects of laser light, magnetic waves, or heat to improve the delivered drug effect at tumour sites [31]. Therefore, combining these external effects with NPs can create a foundation for more innovative approaches exemplified by photodynamic [32], photothermal [33, 34], magnetic [35], and neutron-capturing systems [36]. These multi-disciplinary approaches undoubtedly provide additional armoury to enhance the effect and selectivity of cancer treatment.

NPs are commonly directed to tumour sites through active or passive targeting. Passive targeting represents the ability of NPs to accumulate at cancer sites due to tumour-associated conditions like inflammation and hypoxia [37]. Matsumura and Maeda [38] described the phenomenon of the Enhanced Permeation and Retention (EPR) effect during cancer, which contributes to the accumulation of NPs in cancer tissues. Whereas active targeting relies on ligand-receptor binding that improves selective accumulation at the targeted sites and thus distinguishes between cancerous and healthy tissues [39]. Active targeting involves the implementation of one or more targeting moieties conjugated to the NP surface, which interact precisely with antigens or receptors that are either uniquely expressed or overexpressed on tumour cells compared to normal tissues [40]. This concept was initially tested with antibodies grafted on lipid NPs' surface, known as liposomes [41].

Several passively targeted NPs have been used over the past two decades; nevertheless, none of their actively targeted counterparts have progressed through clinical trials [42]. Additionally, over 40,000 published articles have focused on active targeting strategies in the last decade, yet we still need to address several technical challenges [42]. These include low drug-loading efficiency and solubility, poor ability to cross *in vivo* barriers, low tumour targeting, penetration, and *in vivo* unpredictability. At the same time, additional problems arise from non-specific interactions of hydrophobic compounds, unfavourable ones bio-distribution, and adverse drug release profiles [43]. Consequently, the NP type should be carefully selected to suit each specific cancer type. Likewise, the conju-

gated molecules should be attached to NPs using a suitable linker to ensure the high stability of the conjugates at the site of action.

The Food and Drug Administration (FDA) has approved a selection of polymers for the synthesis of NPs for cancer immunotherapy, such as polyethylene glycol (PEG), poly (lactide-o-glycolic) acid, and chitosan, attributed to their biocompatibility, non-toxicity, and biodegradability [44]. For instance, PEGylating NP surfaces can improve their hydrophilic properties, promote characteristics to avoid their recognition by the immune system, and increase the chance of targeting the desired cells [45]. Polymer-based nanoparticles are the most popular systems in cancer immunotherapy [46]. Liposomes were discovered in 1965 and were the first approved class of therapeutic NPs for cancer treatment [47]. The lipid features, pharmacological properties of liposomal formulations, and their application's clinical studies have been extensively reviewed in other articles [48 - 51].

TYPES OF LIPID-BASED NANOMATERIALS

The main lipid-based nanomaterials include liposomes, niosomes, micelles, solid lipid nanoparticles, and nanostructured lipid carriers.

Liposomes

Bangham first described liposomes in 1965, where they were found to display several dimensional, structural, and functional properties similar to that of biological membranes [52]. Since then, liposomes have been established as vehicles for drug delivery [53] and have been extensively studied. Liposomes are the most studied lipid-based system investigated and have been the first to achieve clinical approval [47, 54, 55].

Liposomes are enclosed vesicular structures which form spontaneously, by self-assembly of lipids upon hydration, into colloidal particles consisting of a lipid bilayer surrounding an aqueous core [56]. The lipid bilayer is arranged to shield the hydrophobic tail moieties from the aqueous environment within the bilayer space by the hydrophilic head groups facing the aqueous phase on both sides [57]. As a result, liposomes serve as versatile carrier vehicles where drugs of varying physicochemical properties can be entrapped in the aqueous core, contained within the bilayer space, or interact with the bilayer surface [58].

Phospholipids, either glycerophospholipids or sphingomyelin, are the main lipid constituent of liposomes. Phosphatidylcholine (PC), also known as lecithin, is the most popular glycerophospholipid used in liposome formation, along with others such as phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol

(PI) and phosphatidylethanolamine (PE) [59]. The inclusion of cholesterol into the liposomal structure helps to improve bilayer fluidity, reduce permeability and reduce liposome identification by plasma proteins and subsequent uptake [60].

Different criteria have been used to classify liposomes; the most predominant is size and lamellarity. Barba *et al.* (2019) classified them into unilamellar vesicles (UVs), further subclassified into small (SUVs, 20-200 nm), large (LUVs, 0.2-1 μm) and giant (GUVs, >1 μm), and vesicle with multiple bilayers including multilamellar vesicles (MLVs, 0.5-5 μm) in which the bilayers are arranged in a concentric manner and multivesicular vesicles (MVVs) consisting of a non-concentric arrangement of close-packed vesicles [59].

The most basic, conventional liposomes can be anionic, cationic, or neutral depending on the charge of the phospholipid used [61]. However, once administered into the blood circulation, these liposomes are subjected to rapid clearance through opsonisation by plasma proteins and subsequent recognition and uptake by the MPS. This can be limiting if a drug is required to be delivered to sites beyond the MPS, such as solid tumors. Functionalising conventional liposomes by attaching a hydrophilic carbohydrate or polymer to their surfaces, most likely a lipid derivative of polyethylene glycol (PEG), prolongs their circulation time [62, 63] and have been termed as long-circulating, sterically stabilised, PEGylated, or stealth liposomes. These liposomes deliver their payload through passive targeting, discussed in later sections. Other types of functionalised liposomes include ligand-targeted liposomes such as immunoliposomes [61] and stimuli-responsive liposomes such as pH-sensitive liposomes, thermal-sensitive liposomes, and magnetic liposomes [64].

Niosomes

Niosomes, also known as nonionic surfactant vesicles, are colloidal particles structurally and functionally similar to liposomes formed by the self-assembly of nonionic amphiphiles upon lipid hydration into vesicular structures [65]. They were first described and used by researchers in the cosmetic industry [66], but with the discovered potential of liposomes in drug delivery, the motivation to use niosomes as drug carriers was also prompted.

As with liposomes, niosomes too are composed of a lipid bilayer surrounding an aqueous core and can therefore be loaded with drugs of different physicochemical properties. They are also classified similarly as liposomes based on size and lamelllarity into SUVs, LUVs, and MLVs [67]. They can also be conventional or functionalised to be sterically stabilised, ligand-targeted, or stimuli-responsive [68].

However, niosomes are considered cheaper alternatives to liposomes as they have less stringent storage and handling requirements, thereby offering greater stability and comparable toxicity [65, 69, 70]. The various types of nonionic surfactants used in niosome formation include alkyl ethers, alkyl esters, and alkyl amides fatty acid [71]. Like liposomes, cholesterol is included in niosomal composition, providing the same benefits by reducing bilayer fluidity, improving entrapment efficiency, and reducing membrane permeability [72, 73]. Charged lipids can also impart electrostatic retention between vesicles, preventing aggregations [73].

Micelles

Micelles are small colloidal structures formed from the self-assembly of amphiphilic molecules, ranging in size between 5-100 nm [74]. In aqueous media, micelles are formed spontaneously into monolayer structures where the hydrophilic heads are arranged to face the aqueous phase and protect the hydrophobic tails that form the inner core. Depending on drug solubility they can be entrapped in the hydrophobic core, adsorbed onto the micelle surface, or dispersed between the surfactant molecules [75]. Depending on the type of amphiphilic molecules, the micelles may be divided into lipid micelles, polymeric micelles, or lipid-polymeric hybrid structures [54]. Micelles can be modified to be PEGylated, ligand-targeted, or stimuli-responsive [76].

Solid Lipid Nanoparticles

A new generation of lipid-based nanomaterials have been presented as better alternatives that combine the advantages and overcome the major limitations of traditional colloidal systems such as liposomes and polymeric NPs [77]. These nanomaterials are based on solid lipids and include solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), and lipid-drug conjugates (LDCs) [78].

SLNs, introduced in 1991, are colloidal particles (10-1000 nm) consisting of a solid lipid matrix at physiological temperature and can entrap both lipophilic and hydrophilic drugs either in dissolved or dispersed form [79]. The solid matrix comprises biodegradable and biocompatible lipids of natural or synthetic origin, including mono-, di- or triglycerides, complex glyceride mixtures, fatty acids, waxes, and steroids [81]. The lipid matrix is stabilised in the aqueous environment by a coating of surfactants or polymer, and in some cases, a co-surfactant can be added as well [82]. Drugs are incorporated into SLNs based on one of three models: they can be homogeneously distributed throughout the matrix (solid solution), concentrated in the outermost portion of the lipid matrix forming a drug-free lipid core (drug-enriched shell), or concentrated in the core of the matrix (drug-enriched core) [83].

However, drug loading capacity is poor due to reduced encapsulation space because the lipids are compactly packed in SLNs in an ideal crystalline structure. Furthermore, polymeric transition during storage can lead to drug expulsion from the carrier system [83]. As a result, NLCs were developed from SLNs by adding liquid lipids to the solid lipids to improve drug loading capacity and prevent lipid crystallisation and subsequent drug expulsion during storage [80]. Liquid lipids used in NLCs include glyceryl tricaprylate, ethyl oleate, isopropyl myristate, and glyceryl dioleate [81].

METHODS OF PREPARATION OF LIPID-BASED NANOMATERIALS

Several approaches have been developed for the synthesis of lipid-based nanomaterials, which are used for the diagnosis, detection, or treatment of cancer. The selection of the appropriate method depends on the physicochemical properties of compounds within the nanoparticles. This section will describe the methods and their most significant characteristics according to their energy requirements.

High-energy Methodologies

High-Pressure Homogenization (HPH) Method

Firstly, the drug is dissolved or dispersed in the melted lipid to obtain a liquid formulation. The high operative pressure pushes the liquid formulation through a micrometric cavity, forming a suspension of homogenised lipid particles with reduced final size [77]. It can be performed either at elevated temperature (hot homogenisation) or below room temperature (cold homogenisation) [84] to produce excellent product characteristics. The principal limitation of this technique has been the high consumption of energy and the polydispersity of the final formulations. Over time, the HPH approach has shown some modifications/ improvements to overcome its original limitations; in this way, narrowest droplets distributions can be produced keeping stable the pressure by alternative concepts such as microfluidics.

Microfluidics is a form of a high-pressure homogenizer. The main difference with the conventional method is the shear rate applied at constant pressure; this is possible using a device or disruption unit with fixed geometry microchannels rather than valves [85].

The key component in this technology is the device used to make the particles. Microfluidic devices are small-scale circuits that consist of several channels (with dimensions of tens to hundreds of micrometres) with adjustable mixings of small volumes of several fluids [86, 87]. Fluid flow is established by external sources

such as manual/digital peristaltic pumps or syringe pumps. There are two parameters to control the composition and size of the final formulation: Flow Rate Ratio (FRR) and the time settings for both inlet ports. The materials selected to make the NP, which encapsulates the drug, can be from long-chain fatty acids to phospholipids or mixtures of glycerides. Some studies have shown reliable outcomes and synergistic effects of the combination lipids-drug and improved the loading capacity and stability of the combinations [70, 88].

High-speed Homogenisation

The emulsion is created by external mechanical energy supplied by rotor mixers or high-speed stirrers. The speed used is between 6000 – 24,000 rpm [89]. The melted lipid and the surfactants in the water phase are mixed at the same temperature under constant stirring. In this way, an oil bath could be used to maintain the temperature until the particles are formed.

Higher stirring rates reduce the polydispersity but do not show significant reproducibility when the process is scaled up [90].

Ultrasonication

The most important parameters for the ultrasonication method are frequency, ultrasonication time, and power of irradiation. These parameters are related to the diameter of the probe tip, and they are tabulated according to the manufacturer's recommendations of the ultrasonicator. The gradual temperature reduction of the final emulsion below the crystallisation temperature of the lipid yields a lipid NP dispersion [91].

Emulsion preparations by ultrasonic irradiations are very reproducible, but heat generation and potential metal contamination due to ultrasonication cause a significant problem with this method, so it is not easily scalable for the industry [92].

Low-energy Methodologies

Spontaneous Emulsification Method

This method is based on the study of phase equilibria between the water, oil (lipids) and surfactant [93]. An essential tool used to study and understand these emulsions is the ternary-phase diagram [94], where the compositions of the three components expressed in terms of the percentages are plotted; The most significant challenge for this method is achieving homogeneous and reproducible spontaneous emulsions [95].

Membrane Emulsification

This method employs a cylindrical membrane module: internal water flow containing the surfactant allows the formation of small droplets when the melted lipid is pressed through the membrane's pores. These droplets are detached from the membrane pores by tangential water flow [96]. The method is scalable, and the particle size can be tuned using membranes with different pore sizes [95]. However, clogging of the membrane reduces its industrial use.

Phase Inversion Temperature (PIT)

This method exploits the ability of specific temperature-sensitive nonionic polyethoxylated surfactants to change their affinities for water and oil as a function of temperature [97]. Oil in water (o/w) emulsions can be obtained due to changes in the surfactant's spontaneous curvature at the oil-water interface and some degrees below the phase inversion temperature PIT [98].

This technique is specific to thermal-sensitive surfactants such as polyethylene oxide (PEO) derivatives, thereby strongly restricting the choice in surfactants [99].

Coacervation

This is a simple method where the precipitation of lipid NPs is produced by acidification (or coacervation) of a micellar solution of fatty acid alkaline salts in the presence of an appropriate amphiphilic polymeric stabilising agent [100]. Using this technique, lipophilic, hydrophobic ion pairs of hydrophilic drugs and even thermosensitive drugs can be incorporated without using solvents. Therefore, it is suitable to scale up if the encapsulated drug is not pH-sensitive [101].

Double Emulsion Method

This technique has been more popular in encapsulating water-soluble anticancer drugs including peptides [95]. Double emulsions have been prepared using two-step emulsification, where the initial step of making the primary emulsion involves using a low hydrophilic-lipophilic balance (HLB) surfactant in case of w/o/w emulsion and a high HLB surfactant in case of o/w/o emulsion. In the second step, a primary w/o emulsion is dispersed in a continuous aqueous phase containing high HLB or hydrophilic surfactant to form w/o/w double emulsion [102].

Methods Based on Using Organic Solvents to Stabilise pH- or Thermosensitive Drugs and Increase their Bioavailability

These can be divided into solvent injection [103], solvent evaporation from emulsions [104], and solvent diffusion from emulsions [105]. These methods require an additional step to remove the solvent from the final preparation, but this step does not ensure that the total solvent is removed; indeed, the residual organic solvent may remain in the final preparation. Moreover, pharmaceutical manufacturers aim to minimise the number and amount of solvents applied in drug production for toxicological reasons. Thus, these methods are the least attractive for the pharmaceutical industry [106].

Methods Based on Supercritical Fluid

These methods solubilise a drug easily by a relatively small change in pressure. The most popular fluids used are carbon dioxide or nitrogen due to their relatively low cost and non-toxicity [107]. There are four methods in this category: Rapid Expansion of Supercritical Solution (RESS) [108], Gas Anti-Solvent (GAS) [109] Process, particles from Gas-Saturated Solution/suspensions (PGSS) [110], and Supercritical Fluid Extraction of Emulsions (SFEE) [111]. These supercritical fluid technologies have great advantages for the environment as they pose no air or water pollution risks [112], although it is still an expensive method for mass production. Additional technological development would be needed for industrial scale-up.

Drying Methodologies

After any of the methods previously described forms the emulsion/dispersion or suspension, one of the following drying technologies can be applied to get powdered lipid-based particles with better storage stability.

Nano-spray-drying

This vibration mesh technique can spray very small quantities of liquids (emulsion, suspension or dispersion) and dry them with a laminar stream of hot drying gas. This method provides NPs in powder form in a short processing time. The encapsulation efficiency and the size of the particles are significantly affected by the spraying parameters, the design of the nozzle, drying temperature, and the liquid feed's physicochemical properties [113]. The main constraints of this technique are that it cannot be scaled up, and the yield of the process is low [114].

Freeze-drying

Freeze-drying or lyophilisation is a water removal process developed primarily to encapsulate heat-sensitive compounds with no presence of solvents. In this process, the reduction of the pressure (vacuum) and the addition of heat allow the sublimation of the frozen water, preserving the physical form of biological compounds. The main problems of freeze-drying include their use with aqueous emulsion, long process times, and high energy consumption by the vacuum pump [115].

Variosol

This is a solvent-free technology able to produce microparticles exploiting liquid or near-critical CO_2 properties. It is based on the capability of rapidly expanding liquid-dense CO_2 to adsorb heat (Joule-Thomson effect) while generating a pressure gradient, thus simultaneously inducing rapid cooling, controlled solidification, and atomisation of fluid materials during spraying [116].

CLINICAL APPLICATIONS OF LIPID-BASED NANOMATERIALS

The application of NPs in treating various diseases has been extensively studied with a greater focus on cancer. The clinical approval of the first NP in the form of liposomal doxorubicin (Doxil) in cancer treatment has paved the way for the approval of several other lipid-based formulations, and many more are undergoing different phases of clinical development. Those in clinical trials are either approved nanoformulations (*e.g.*, for another cancer type or in combination with other therapies) or newly developed ones [117]. The clinical applications of lipid-based nanomaterials in cancer treatment that have been explored include chemotherapy [50], gene therapy [59], immunotherapy [118], and radiotherapy [119] delivery systems, and some of them are highlighted here.

Talidox (TLD-1) is a new PEGylated liposomal formulation of doxorubicin developed by InnoMedica as an improved version of the already approved Doxil (Myocet and Caelyx/Doxil). TLD-1 was developed as a solution to the high incidence of Palmar-Plantar Erythrodysethesia (PPE; also known as a hand-an--foot syndrome) associated with the use of Caelyx despite its ability to prevent doxorubicin-related cardiotoxicity (InnoMedica Talidox Brochure, 2018; retrieved from https://www.innomedica.com/wp-content/uploads/2018/04/Talidox.pdf). Preclinical studies demonstrated the superiority of TLD-1 compared to free doxorubicin and Caelyx in terms of enhanced cytotoxicity and antitumour activity with reduced toxicity [29, 120]. This has prompted a Phase I trial in patients with advanced solid tumours (NCT03387917).

Annamycin is an improved new-generation lipophilic anthracycline antibiotic characterised by avoidance of multidrug resistance and lack of cardiotoxicity in contrast to doxorubicin [121]. Moleculin Biotech, Inc. has developed a liposomal formulation of annamycin studied in two different Phase I/II trials in patients with refractory or relapsed acute myeloid leukaemia (NCT03315039; NCT03388749).

The bioavailability of mytomycin C was enhanced by delivering it as a lipid-based prodrug in PEGylated liposomes known as Promitil® [122]. A stable liposomal formulation is produced by encapsulating the prodrug within the bilayer, which is only released as the active drug by the thioredoxin-thioreductase system, which is overexpressed in tumours thereby improving tumour localisation [123]. Promitil, developed by Lipomedix Pharmaceuticals Inc., has been studied in a Phase I trial to treat patients with solid tumours (NCT01705002). A different Phase I study of Promitil combined with external beam radiotherapy is also being investigated in patients with solid tumours (NCT03823989).

Stimuli-responsive liposomes have also been investigated as a means to improve drug bioavailability. One example is a heat-responsive liposomal formulation of doxorubicin known as lyso-thermosensitive liposomal doxorubicin (LTLD) or ThermoDox developed by Celsion Corporation [124]. LTLD is PEGylated consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxypolyethylene-glycol-2000, and is designed to release its payload at temperatures above 39.5 °C within seconds [125]. Following its administration by intravenous infusion, local heat is applied, which is beneficial in both maximising liposomal localisation in tumour sites due to increased vasculature and triggering doxorubicin release [125]. Local heating can be obtained by radiofrequency ablation (RAF), microwave hyperthermia, or high-intensity focused ultrasound. ThermoDox has reached several clinical phases of the study. The OPTIMA trial (completed Phase 3) studied the combination of LTLD with standardised RFA in treating non-resectable hepatocellular carcinoma (NCT02112656). Another study also combining LTLD with RFA has completed Phase I in cases of primary and metastatic liver tumours (NCT00441376). The TARDOX trial has completed Phase 1 and combines LTLD with focused ultrasound in patients with primary or secondary liver tumours (NCT02181075) and has shown promising results. The DIGNITY trial has completed Phase I/II evaluating LTD combined with microwave hyperthermia in treating local-regional recurrent breast cancer (NCT00826085). Currently patients with metastatic breast cancer are being recruited in a Phase I trial to study LTLD combined with magnetic resonance guided high intensity focused ultrasound (MR-HIFU) and cyclophosphamide as part of the i-GO study (NTC03749850). LTLD and MR-

HIFU will also be investigated in a Phase I study currently recruiting children with relapsed and refractory solid tumours (NCT02536183).

Polymeric micelles have successfully reached clinical trials as delivery vehicles for chemotherapeutic drugs. Genexol PM developed by Samyang Biopharmaceutical Corporation is a novel polymeric micellar formulation of paclitaxel [126]. Genexol PM has been investigated in many clinical trials including pancreatic cancer either alone (Phase II; NCT00111904) or combined with gemcitabine (Phase I/II: NCT00882973), urothelial cancer (Phase II; NCT01426126), in non-small cell lung cancer combined with cisplatin (Phase II; NCT 01023347) and with gemcitabine (Phase II; NCT01770795), in ovarian cancer combined with carboplatin (Phase I; NCT0087725 and Phase II; NCT01276548), in advanced head and neck cancer combined with cisplatin (Phase II; NCT01689194). A novel sustained-release micellar formulation of cisplatin (NC-6004) was developed by NanoCarrier Co., Ltd. to overcome cisplatin-associated toxicities [127]. NC-6004 has been studied in combination with gemcitabine in cases of pancreatic cancer (Phase I/II; NCT00910741 and Phase III; NCT02043288) and solid tumours (Phase I/II; NCT02240238) and in combination with fluorouracil (5-FU) and cetuximab to treat head and neck cancer (Phase I/II: NCT03109158).

Significant progress in gene delivery was achieved with the first lipid-based nanomaterial, Onpattro, to be approved in cancer treatment and many more are in the pipeline. SynerGene Therapeutics, Inc. has developed cationic liposomal formulations; SGT-94 delivering plasmid DNA encoding the tumour suppressor gene RB94 [128] and SGT-53 delivering plasmid DNA encoding the normal human wild type p53 suppressor gene [129]. Cationic liposomes, composed of DOTAP and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), are conjugated with an anti-transferrin (TfR) single-chain antibody fragment and target tumours by binding to TfR, which is overexpressed in cancer cells [130]. A Phase I study with SGT-94 in patients with solid tumours has been completed (NTC01517465). A Phase Ib trial of SGT-53 combined with docetaxel in patients with solid tumours has also been completed (NCT00472613). An ongoing Phase 1 trial investigates SGT-53 alone and in combination with topotecan and cyclophosphamide in children with recurrent or refractory solid tumours (NCT02354547). Patients with metastatic pancreatic cancer are also being recruited for a Phase II study combining SGT-53 with gemcitabine/nab-paclitaxel (NCT02340117).

The delivery of the tumour suppressor gene TUSC2(FUS1), which is inactivated in 100% of small cell lung cancer and 82% of non-small lung cancer cases, was investigated in the treatment of lung cancer by complexing the gene with cationic

liposomes consisting of DOTAP and cholesterol [131]. This NP delivery system, known as ONCOPREX® by Genprex, Inc., was developed initially with the University of Texas MD Anderson Cancer Centre and the National Institute of Health (Genprex website retrieved from https://www.genprex.com/technology/oncoprex-nanoparticle-delivery-system/). The synergistic effect of this liposomal formulation with erlotinib is currently being investigated in an ongoing PhaseI/II trial in Stage IV non-small cell lung cancer (NCT01455389).

MTL-CEBPA developed by MiNa Therapeutics, Ltd. is the first-in-class small activating (saRNA) oligonucleotide delivered in the SMARTICLES® liposomal product [132, 133]. The NP formulation is designed to upregulate CEBPA (CCAAT/enhancer-binding protein alpha) expression in the liver to treat cirrhotic hepatocellular carcinoma [134]. The potential of MTL-CEBPA in acute and chronic, and inflammatory diseases is also reported [135]. Promising results of a first-in-human Phase I trial in cases of advanced HCC using MTL-CEBPA alone has prompted the OUTREACH trial, a Phase Ia/b trial currently investigating the combination of MTL-CEBPA with sorafenib in advanced HCC (NTC02716012). The TIMEPOINT trial recruits patients with advanced solid tumours in a Phase Ia/b study combining MTL-CEBA with pembrolizumab (NCT04105335).

A neutral liposomal formulation of DOPE was developed to deliver the EphA2-siRNA (EPHARNA) to target cancer cells overexpressing the EphA2 receptor [136]. A Phase I trial is currently recruiting to investigate the formulation in advanced or recurrent solid tumours (NTC01591356).

The liposomal formulation BP1001, entrapping Grb2 antisense oligonucleotide, was developed by Bio-Path Holdings to downregulate the growth factor receptor-bound protein 2 (Grb2) responsible for tumour progression [137, 138]. A Phase I/b trial investigating BP1001 in patients with refractory or replaced acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), Philadelphia-chromosome-positive chronic myeloid leukaemia (CML), or myelodysplastic syndrome (MDS) has been completed (NTC01159028) with favourable results [137]. A Phase II trial is currently recruiting to investigate the combination of BP1001 with venetoclax plus decitabine in patients with AML (NTC02781883).

Cancer immunotherapy using lipid-based nanomaterials, including vaccine delivery, have also been explored [118, 139]. OncoQuest-CLL developed by XEME Biopharma Inc., is a liposomal product delivering a vaccine composed of whole-cell tumour lysates with IL-2 for early intervention in asymptomatic chronic lymphocytic leukaemia (CLL) [140]. The patient's leukaemia cell lysate and a recombinant human IL-2 are delivered in multilamellar liposomes providing personalised cancer vaccines (XEME Biopharma website retrieved from

https://www.xemebiopharma.com/personalized-vaccines). A Phase Ib trial to study the vaccine's safety and efficacy in patients with previously untreated CLL is ongoing (NCT01976520). An mRNA vaccine-loaded liposomal product, W_ova1, against ovarian cancer is being investigated in Phase I of the OLIVIA trial with carboplatin and paclitaxel (NCT04163094). The IMMUNOCERV trial is a Phase IIA trial investigating the liposomal multi-peptide vaccine (PDS0101) against human papillomavirus (HPV16 E6/E7) combined with cisplatin and radiation therapy in patients with Stage IB3-IVA cervical cancer (NTC04580771).

The applications of lipid-based materials in cancer treatment have also been explored in the delivery of radiotherapy. Brachytherapy by convection-enhanced delivery of liposomes entrapping the radionuclide rhenium-186 was investigated to treat glioblastoma, demonstrating promising preclinical results [119]. The liposomal product 186RNL, developed by Plus Therapeutics, is currently being studied in Phase I/II of the ReSPECT trial in patients with recurrent or progressive glioma (NCT01906385).

CLINICALLY APPROVED LIPID-BASED NP MEDICINES

The FDA's first approved product of nanomedicines was Doxil® which was approved in 1995 for the treatment of ovarian cancer and AIDS-related Kaposi's sarcoma [60, 141 - 143]. Doxil®, a PEGylated liposomal doxorubicin, was a liposomal formulation attached with polyethylene glycol (PEG) chains on the liposome surface (PEGylation) [141, 144, 145]. This formulation was produced to minimise the limitations of the conventional non-PEGylated liposomes regarding short circulating half-life due to rapid clearance by macrophages. Also, Doxil® was proven effective in reducing cardiotoxic side effects of doxorubicin (DOX) treatment and enhanced drug levels in malignancies compared to free DOX [142, 144, 146]. Other liposomal formulations with DOX were designed to be more tolerable and more effective than free DOX, such as Lipo-Dox® [142]. Moreover, Myocet®, co-encapsulation of DOX and cyclophosphamide in a liposome was approved by the European Medicines Agency (EMA) in 2000 and indicated for the treatment of metastatic breast cancer [141, 142, 146, 147]. The PEGylated liposomal carrier was quickly adopted to deliver other drugs such as Abelcet® and Ambisome®, an amphotericin B to treat invasive fungal infections [144, 146, 148]. Amphotericin B is used to treat invasive fungal infections and acts through the interaction of drugs and sterols in the cell membrane of susceptible fungi, with a resulting change in membrane permeability [142, 146]. The liposomal formulations were presented as AmBisome®from NeXstar Pharmaceuticals, Inc. (now Astellas Pharma, Inc.); lipid complexes such as Abelcet® from Enzon Pharmaceuticals (now Sigma Tau Pharmaceuticals, Inc) [142, 148].

In the 2000s, there has been a growing number of trials and approvals using liposomal delivery. Liposomes encapsulated with a photosensitiser for photodynamic therapy were introduced [148]. Visudyne®, a product of Novartis AG, Switzerland, is a unilamellar phospholipid vesicle based on DMPC and egg phosphatidyl glycerol (EPG) delivering verteporphin (VPF), a synthetic chlorine-like porphyrin [142, 144, 146]. Visudyne® is the first light-activated drug available for treating patients with predominantly classic subfoveal choroidal neovascularisation due to age-related macular degeneration (AMD) [146, 148]. This formulation was designed to eradicate the abnormal blood vessels in the eye-related to conditions such as wet macular degeneration [144]. Paclitaxel (PTX) is a well-established therapeutic drug used for ovarian cancer therapy. PTX in liposomal formulations such as Lipusu®, a formulation approved in China (Sike Pharmaceutical Co. Ltd.), has been developed [142]. Subsequently, more approved liposomal formulation products have become available to treat cancer and different diseases. For instance, Exparel®, a bupivacaine liposomal injection form, is a multivesicular liposome based on Depofoam™ technology used for pain control [146, 149]. Pacira Pharmaceuticals, Inc. and FDA-approved developed Exparel® for local surgical site injection for post-operative pain after maemorrhoidectomy and bunionectomy [142, 149, 150]. DepoDur®, liposome encapsulated morphine sulphate has been developed by SkyePharma, San Diego, CA for administration before surgery or following clamping of the umbilical cord during a caesarean section [142, 146].

The first product based on a liposomal vaccine or virosome technology was Epaxal® which Crucell Berna Biotech, Switzerland patented. Epaxal® is a virosome-adjuvanted vaccine for hepatitis A (HAV) with high tolerability [142, 146]. This vaccine lacks aluminium salts and thiomersal, allowing intradural or subcutaneous administration due to fewer adverse local effects than conventional aluminium-adsorbed vaccines. Virosomes are spherical vesicles composed of phospholipids, lecithin, phosphatidylcholine, and phosphatidylethanolamine that contain viral envelope glycoproteins intercalated in the phospholipid bilayer membrane. The lipid components of Epaxal® virosomes are (DOPE) and DOPC. The hepatitis A vaccine (HAV) vaccine Epaxal® is based on formalin-deactivated RG-SB strain HAV particles combined to the surface of virosomes. The RG-SB HAV strain was produced on an MRC-5 human diploid cell culture, and then the virus was purified from disrupted cells by ultrafiltration. HAV was deactivated by treatment with formalin and then attached to the virosome surface. This structure facilitates the delivery of the HAV antigen to immunocompetent cells owing to the properties of fusion-active glycoproteins [51]. A clinical trial study demonstrated that more than 95% of subjects were protected from HAV infection for more than 20 years [146, 151]. Inflexal® V is also a virosomal-adjuvanted vaccine that contains the *influenza* virus. It is produced from the double

membrane of lecithin-phospholipid liposomes loaded with haemagglutinin surface molecules of *influenza* viruses after preparation of *influenza* surface glycoproteins, neuraminidase (NA), and hemagglutinin (HA) by detergent treatment. The superior immunogenicity of Inflexal® V has shown statistically significant improvement above conventional *influenza* vaccines [146, 152, 153]. This formulation was patented by the Crucell Berna Biotech, Switzerland, and approved by EMA in 2008.

In 2012, Marqibo®, a vincristine (VCR) sulfate nanoliposomal injection form produced by Talon Therapeutics, Inc. USA, was FDA-approved for the treatment of adult patients with Philadelphia chromosome-negative (Ph^-) acute lymphoblastic leukemia (ALL) [141, 146, 148]. VCR is a semi-synthetic chemotherapeutic agent encapsulated in an aqueous interior core of sphingomyelin/cholesterol nanoliposomes to overcome the dosing, pharmacokinetics, and pharmacodynamics limitations of non-liposomal VCR [146, 148]. Marqibo® has proved to be safe and shown tolerability, with enhanced vincristine cell uptake, penetration, and concentration in tissues and organs with fenestrated vasculature or involved in the MPS [141, 142, 146].

Since 2015, three new liposomal formulations have been approved for clinical use by FDA. Onivyde® (irinotecan liposome injection), a product produced by Merrimack Pharmaceuticals Inc., was approved by the FDA and the EMA in 2015. Onivyde® is a PEGylated nanoliposomal hydrochloride irinotecan formulation for the treatment of metastatic pancreatic adenocarcinoma (mPAC) in combination with 5-FU and leucovorin (LV) in patients previously treated with gemcitabine-based therapy [142, 143, 148]. In general, there are still very few options for these patients, and no consensus of care has been established. It was shown that patients given Onivyde® in addition to 5-FU/LV were shown to have a higher survival rate [142, 146]. Moreover, CPX-351 (Vyxeos™), FDA-approved in 2017, is also a liposomal product delivering cytarabine and daunorubicin for the treatment of acute myeloid leukemia (AML) [142, 148, 154]. Liposomal daunorubicin has proven effective with a low cardiac toxicity profile in an increased anthracycline dose in older patients, children, and adolescents. It also increases the plasma half-life and increases drug accumulation within the bone marrow [142].

The most recently approved liposomal drug carrier is ONPATTRO® (2018) or Patisiran [142]. ONPATTRO® is a siRNA-delivering liposome developed and marketed by Alnylam for silencing of a specific gene responsible for the expression of transthyretin (TTR), which can cause hereditary transthyretin amyloidosis (hATTR) [142, 143, 155]. Thus, Patisiran comprises a TTR mRNA-specific siRNA formulation [155, 156]. It is also the first clinically approved

example of an RNAi therapy-delivering NP that can be administered intravenously. It is also the first therapeutic RNAi approved by the FDA [143, 155]. Clinical data have shown a potent and sustained knockdown of TTR expression. The patients who received the RNA had better outcomes on measurement of polyneuropathy, including muscle strength, sensation (pain, temperature, numbness), reflexes, and autonomic symptoms including blood pressure, heart rate, and digestion compared to those receiving the placebo infusions. However, it still has side effects, and there has been little evidence of safety concerns about platelets, renal function, or liver enzyme elevations [142]. Additionally, it was suggested that patisiran might stop or possibly reverse the progression of hATTR [142, 157].

From this evidence, liposomal drug delivery has become a valuable tool for clinical medicine, and this platform will continue to evolve into next-generation nanomedicines with improvements in nanotechnology. Liposomal formulation products that have been approved for clinical use are shown in Table **1**.

Table 1. Approved liposomal formulation products for clinical use.

Trade Name	Active Agent	Indications	Status
CPX-351 (Vyxeos™)	Daunorubicin and cytarabine	Acute myeloid leukemia	Approved by FDA in 2017
Onivyde	Irinotecan, fluorouracil and folinic acid	Pancreatic adenocarcinoma	Approved by FDA in 2015
Marqibo	Vincristine	Non-Hodgkin's lymphoma and leukemia	Approved by FDA in 2012
Exparel	Bupivacaine	Pain management	Approved by FDA in 2011
Mepact	Mifamurtide	Osteosarcoma	Authorised for use in the European Union
Genexol-PM	Paclitaxel	Breast, lung, and ovarian cancer	Approved in Korea and marketed in Europe in 2007
Lipusu	Paclitaxel	Gastric, ovarian, and lung cancer	Approved by FDA in 2005
Lipo-Dox	Doxorubicin	Breast and ovarian cancer	Approved by FDA in 2012
Myocet	Doxorubicin + cyclophosphamide	Metastatic breast cancer	Approved by EMEA in 2000
Visudyne	Verteporphin	Ocular histoplasmosis	Approved by FDA in 2000

(Table 1) cont.....

Trade Name	Active Agent	Indications	Status
Abelcet	Amphotericin B	Invasive fungal infection	Approved by FDA in 1995
Doxil	Doxorubicin	Kaposi's sarcoma	Approved by FDA in 1995
AmBisome	Amphotericin B	Invasive fungal infection	Approved by FDA in 1997
ONPATTRO®	Patisiran (siRNA)	Hereditary transthyretin amyloidosis	Approved by FDA in 2018

LIPID-BASED NANOMATERIALS IN DISEASES DIAGNOSIS

Nanotechnology is a useful approach quickly growing in several research fields, especially biomedical sciences. It provides various application forms of NPs, which offer an opportunity to achieve a better diagnosis, therapy, and advanced multifunctional nanomedicines. NPs represent an innovative tool that incorporates molecular target ligands, active molecules, and imaging agents for disease diagnosis and treatment assessment [144, 148]. Among the nanocarriers, lipid-based NPs are mainly attractive for biomedical use due to their beneficial biocompatibility, ease of synthesis, and encapsulation of multiple functional substances [158]. The application of lipid-based NPs in medical imaging and diagnosis has been vastly investigated in SLNs, nanostructured lipid carriers (NLCs), and liposomes [145, 158, 159].

SLNs consists mainly of solid lipid with relatively low toxicity and stabilised with surfactant. The most common lipids used to prepare SLNs include highly purified triglycerides, fatty acids, and complex glycerides mixtures stabilised by a mixture of surfactants or polymers [158, 160, 161]. SLNs serve as a proficient and multipurpose colloidal drug carrier with useful features such as site-specific targeting, control of drug release, physical stability, ease of manufacturing, and industrial scale-up. Importantly, it also can evade or minimise the degradation of loaded drugs [145]. SLNs have been employed as a pharmaceutical tool to transfer many classes of diagnostic agents such as superparamagnetic iron oxide, technetium-99 (^{99m}Tc), ^{64}Cu, and quantum dots (QDs) [145, 158, 161]. Superparamagnetic iron oxide was encapsulated in SLNs and *in vitro* analysis showed a slow blood clearance of this formulation. *In vivo* magnetic resonance imaging (MRI) study also demonstrated the uptake of SLNs by the central nervous system (CNS), and SLNs were able to bypass the blood-brain barrier. This indicates favourable properties of SLNs for use as a CNS MRI contrast agent [145, 162, 163].

Moreover, it has been reported that SLNs could be radiolabelled with ^{64}Cu for positron emission tomography (PET) imaging [164]. SLNs loaded with copper-specific chelator-conjugated synthetic lipids were then radiolabelled with ^{64}Cu. Their stability was shown over 48 hours and ^{64}Cu circulating in the blood was observed 3 hours after injection. This indicates the potential of ^{64}Cu-SLNs for quantitative determination and *in vivo* PET imaging. In addition, SLN loaded with quantum dots can be used for theranostic applications. Co-encapsulation of paclitaxel-siRNA combination and quantum dots into SLNs showed anticancer activity on human lung carcinoma cells. Meanwhile, the intense fluorescence from quantum dots within SLNs indicated the visualization of the intracellular translocation of SLNs in cancer cells [165]. This suggests the usefulness of SLNs as multifunctional nanocarrier for both therapeutic and diagnostic tools.

NLCs have been developed as an improved novel generation of lipid NPs. The nanostructure of NLCs is a mixture of solid and liquid lipids with a lipophilic core that provides a suitable environment for the encapsulation of hydrophobic drugs [158]. Due to their potential to improve the solubility of the lipophilic drug, NLCs are promising carriers for poorly water-soluble drugs. It has been evidenced that the application of NLCs as a bioimaging agent delivery is used in medical imaging such as computerised tomography (CT) scanning, MRI, optical imaging, and gamma scintigraphy for diagnostic applications [158]. For instance, QDs-loaded NLCs were successfully formulated, and the cellular internalisation of QDs was conserved after NLC encapsulation. QD-NLCs exhibited the highest fluorescence intensity after cellular uptake, and *in vivo* real-time monitoring revealed a persistent signal of labelled tumours for 24 h [166]. The experimental data indicate that this lipid nanocarrier might be a promising delivery diagnostic NP.

The application of liposomes in diagnostic medical imaging is a new and rapidly growing field [158]. The role of liposomes in imaging is to offer detectable differences between pathological and normal tissues and permit appropriate imaging. They can also deliver diagnostic agents for gamma scintigraphy, ultrasonography, and bioimaging [158]. Liposomes can be labelled with diagnostic agents including QDs, gadolinium (Gd) and manganese (Mn) (both aqueous and chelated), radionuclides such as Ga, ^{111}In, and ^{99m}Tc, iodine-based agents, and even gases (echogenic liposomes) [158, 159, 167, 168]. Due to the flexibility of the liposome structure, it allows incorporation of imaging agents into either the bilayer or interior that making them effective carriers for intensification of contrast. Liposomes can encapsulate a wide range of contrast agents to improve the contrast *in vivo* visualization [169 - 171]. Liposomes containing materials such as Gd have shown to be valuable for enhanced tumour diagnosis. Gd's encapsulation or conjugation in liposomal formulation shows long retention time

in the circulatory system, whereas pure Gd is quickly reduced in the intravascular plasma and dispersed extracellularly [172].

Furthermore, liposomes-encapsulating ^{64}Cu show unique imaging characteristics. Due to a protective barrier of the liposome membrane can prevent an exchange of ^{64}Cu with the biological environment, resulting in a high concentration of ^{64}Cu within the liposomes. Therefore, these liposomes provide non-invasive visualization and quantification system appropriate for PET/CT imaging [173]. These applications reveal the increasing use of lipid-based nanomaterials in the field of disease and particular cancer diagnosis and monitoring and treatment by enabling targeted delivery of therapeutic agents to the target tissues.

LIPID-NANOPARTICLES TOXICITY

When designing lipid-based nanoparticles formulation, careful attention must be paid to these nanoparticles potential toxic effects. While there are too many reports for the applications of these nanoparticles, the number of reports that address their toxic effects is limited.

The design of a specific lipid nanoparticles drug delivery system should be associated with careful evaluation of the cytotoxicity of these nanoparticles on different cell lines *in vitro* and on the animal model *in vivo*. For example, solid lipid nanoparticles made from stearic acid or dimethyl-dioctadecylammonium bromide (DDAB) were non-toxic at concentrations around 0.01% [174]. Niosomes nanoparticles made of tween 85 and cholesterol and DDAB were non-toxic at concentrations lower than 156 μg/ml [156]. It is worth mentioning that the IC_{50} will be different for the same formulation on different cell lines. The different uptake abilities can explain this by different cell lines, resulting in different toxicities [175].

In several studies, positively charged lipid nanoparticles are usually associated with higher toxicity than neutral particles. For example, higher hepatotoxicity with elevation in liver enzymes associated with weight loss was reported for mice treated with cationic nanoparticles compared to mice treated with neutral or anionic nanoparticles [176, 177].

Moreover, following intravenous administration of cationic nanoparticles into the mice model, several immune responses were detected, such as type I interferon response with elevated mRNA levels of interferon responsive genes in different subsets of leukocytes. An increase in the pro-inflammatory responses was also detected by expressing the Th1 cytokine such as IL-2, IFN g, and TNF a. This elevation was also higher for cationic nanoparticles than neutral or anionic particles [174, 176].

CONCLUSIONS

Different lipid-based nanomaterials can be effectively used to improve the diagnoses and treatment of various cancer types by enhancing the detection of cancer-specific biomarkers, imaging of tumours and their metastases, specific drug delivery to target cells, and real-time observation of treatment progression. Among these, liposomes represent the most widely used and studied lipid nanoparticles. Several lipid-based nanomaterial medications have been introduced to the market for these purposes. Many others are yet to come after passing their clinical trials or the development stages, which will significantly improve the outcomes of anticancer therapeutics and diagnostics.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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CHAPTER 4

Polymeric Nanomaterials for Cancer Theranostics

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Abstract: Despite global efforts for decades, the number of cancer cases is still on the rise. Although in recent times there has been significant improvement in immunotherapy, chemotherapy remains standard care for cancer patients alongside radiation and surgery. Chemotherapeutic drugs and diagnostic agents (MRI, PET, Ultrasound) lack specificity and often suffer from poor solubility and unwanted biodistribution. This results in unnecessary high dose requirements, systemic toxicity, and compromised quality of life for the patients. Beside therapy, early diagnosis is essential for the successful treatment and cure of cancer patients, just like any other disease. Therefore, a suitable delivery vehicle is always needed for the theranostic agents. Viral vectors are routinely used for the delivery of genetic material. But parallelly, nanoparticles made with biodegradable, non-toxic, and non-immunogenic polymers are often used as a carrier of chemotherapy drugs, diagnostic agents as well as genetic materials. Once decorated with specific ligands, these nanocontainers can deliver cargo molecules to target tissue and organs with high precision.

Keywords: Biodistribution, Cancer, Cargo, Cationic, Chemotherapy, Delivery, Diagnostic, Drug, Encapsulation, Gene, Hydrophobic, Hydrophilic, Immunotherapy, Ligand, Nanoparticle, Polymer, Receptor, Target, Toxicity, Systemic.

INTRODUCTION

Despite significant global efforts into the research and development of cancer therapeutics for decades, the number of cancer cases is expected to increase continuously. It has been estimated that there are currently 18.1 million new cancer cases every year, with 9.6 million deaths per year, accounting for 1 out of 6 deaths globally [1]. Cancer therapies are currently limited to surgery, radiation, and chemotherapy, with very few exceptions of immunotherapy (CAR-T or checkpoint inhibitors). All three methods risk damage to normal tissues or incomplete eradication of cancer. Beside surgery and therapy, a lot of emphasis also needs to be put on the diagnostics of cancer. Just like any other disease, if

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detected early enough, many types of cancer can be cured. Consequently, cancer therapy has become a multidisciplinary challenge requiring close collaboration among clinicians, biological and material scientists, and biomedical engineers.

Though chemotherapy is successful to some extent and remains standard of care for most cancers, the chemotherapy drugs lack specificity. Thus, in the process of killing cancer cells, they also damage healthy tissues leading to systemic toxicity and adverse side effects. It significantly affects the quality of life of the patient. Most chemotherapeutic drugs are administered *via* intravenous or oral route, which leads to rapid clearance and unwanted biodistribution of the drug. Therefore, chemotherapy drugs need to be administered in excess leading to unnecessary side effects [2]. That is why a vehicle with high specificity to cancer cells can circumvent the undesired systemic toxicity of the chemotherapeutic agents and other drugs.

Often that vehicle is a nanoparticle, either polymeric or protein-based. Nanoparticles are submicron-sized particles with diameters ranging from 10 to 1000 nm that can either encapsulate or display a cargo molecule of interest. These nanoparticles can also be decorated with receptor-specific ligands that can deliver the cargo molecule specifically to the desired tissue or organ, curtailing toxicity [3 - 5]. This book chapter discusses the application of polymeric nanoparticles as theragnostic in cancer.

POLYMERIC NANOMATERIALS IN CANCER THERAPY

Delivery of Chemotherapeutics

Every time any new cell is formed, it goes through a usual process to become a fully functioning (or mature) cell. The process involves a series of phases and is called the cell cycle. Chemotherapeutic drugs target cells at different phases of the cell cycle. Understanding how these drugs work helps doctors predict which drugs are likely to work well together. Doctors can also plan how often doses of each drug should be given based on the timing of the cell phases. Cancer cells tend to form new cells more quickly than normal cells, and this makes them a better target for chemotherapy drugs. However, chemo drugs cannot tell the difference between healthy cells and cancer cells. This means normal cells are damaged along with the cancer cells, and this causes side effects. Each time chemo is given, it means finding a balance between killing the cancer cells (to cure or control the disease) and sparing the normal cells (to lessen side effects). The good news is that most normal cells will recover from the effects of chemo over the time. But cancer cells are mutated (not normal) cells, and they usually do not recover from the effects of chemo. Therefore, chemotherapy is good at killing many types of cancer cells (Table **1**).

Table 1. Different types of chemotherapeutic drugs and their mechanisms of action.

Types of Drugs	Mechanism of Action	Name of Few Drugs
Alkylating agents	Stops cell proliferation by DNA damage	Cisplatin, Busulfan, Oxaliplatin, Trabectedin
Antimetabolites	Interference with DNA and RNA by acting as a substitute for the normal building blocks of RNA and DNA.	5-fluorouracil, Capecitabine, Hydroxyurea, Thioguanine
Antitumor antibiotics	Altering DNA inside cancer cells to keep them from growing and multiplying.	Doxorubicin, Epirubicin Idarubicin, Valrubicin
Topoisomerase inhibitor	Interference with topoisomerases	Irinotecan, Topotecan, Etoposide, Teniposide
Mitotic inhibitors	They work by stopping cells from dividing to form new cells	Paclitaxel, Docitaxel, Vinblastine, Vincristine

We will discuss one specific example of Paclitaxel. It is a microtubule-stabilizing agent that triggers polymerization of tubulin, killing cancer cells by disrupting the dynamics necessary for cell division. It has been found to be effective against a wide range of cancers, including head and neck, ovarian, lung, breast, and colon cancers. However, Paclitaxel is a highly hydrophobic drug with extremely low solubility in water and biological fluid (< 0.5 mg/L) [6, 7]. This drug on its own cannot be administered by intravenous injection as it can aggregate in blood vessels leading to embolization. It often shows local toxicity because of high drug concentrations at the site of deposition. This is why paclitaxel needs to be adequately formulated prior to administration.

The currently available formulation for paclitaxel includes the use of Cremophor EL (polyethoxylated castor oil) and dehydrated ethanol. Unfortunately, Cremophor EL is known to be toxic and can cause serious side effects [5]. Alternatively, surfactants could be used to solubilize the drug, but due to their high critical micellar concentration they cannot maintain the nanoparticle architecture. In this regard, thermodynamically stable polymeric micelles with a hydrophobic core and hydrophilic surface could serve as an effective delivery system for poorly soluble drugs [8 - 10].

The biodistribution of a given drug is a major factor for the success of chemotherapy. To minimize off-target toxicity and rapid clearance from the body, anticancer drugs must show sustained, controlled, and targeted release. In theory, these properties could be obtained by developing formulations that are controlled at the nanometer scale. For example, controlled and sustained drug release may be achieved by precisely adjusting the composition of nanoparticle formulations. Improved drug targeting ability could be obtained by functionalization of the

formulation with ligands that can bind to overexpressed receptors (*e.g.*, CD44] on cancer cells.

Delivery of Genetic Material

Alongside delivery of water insoluble chemotherapeutics, cationic lipids and polymers (both synthetic and natural) are also routinely being investigated for the delivery of genetic material (*e.g.*, pDNA, mRNA, tRNA, siRNA) to treat cancer, infectious diseases and genetic disorders [11, 12]. While viral vectors are more extensively studied and some of them are approved by the regulatory agencies and are in commercialization stage, nonviral vectors are far behind. Most of non-viral gene delivery vehicles are in preclinical and early clinical stage. This slow progress can be attributed to lower transfection efficiency associated with nonviral vectors. The efficiency of gene transfer *via* nonviral vectors has been reported to be 1/10th to 1/1000th that of viral vectors. Also, non-viral gene delivery requires amalgamation of chemistry and biology, which can often be a roadblock. As a result, despite lot of manufacturing, mechanism of action and preclinical data in non-viral gene delivery doing rounds, no significant translation to clinic and BLA application or approval has happened [13]. But with the very recent success of the two Covid-19 vaccines from Moderna and BioNtech-Pfizer, the future certainly seems bright for non-viral gene delivery.

In non-viral gene delivery, the cationic polymer or lipid and the gene of interest are manufactured separately. Once mixed, the cationic portion of the vehicle binds to negatively charged nucleic acids and helps to condense the large genes into small nanomaterials called polyplex (for polymers) or lipoplex (for lipids) [14 - 18].

Owing to the net surface positive charge, the lipoplex or polyplex can bind to the negatively charged cell surface, gets encapsulated inside the cell by clathrin or caveolae mediated pathway, ensures endosomal escape (mostly by “proton-sponge” effect), which eventually lets the nucleic acid enter nucleus and produce protein. But these cellular uptakes mechanisms are non-specific. To attain target specificity, the polyplex or lipoplexes can be functionalized with receptor-specific ligands [19 - 22].

Delivery of Diagnostics

Theranostic, a term coined by Funkhouser in 2002, describes any “material that combines the modalities of therapy and diagnostic imaging” into a single entity [23]. In recent years, meaning of theranostics has evolved. Now it is used to describe “therapeutic agents that concomitantly possess imaging capabilities” [24]. The conventional approach to treating patients involves diagnosis of the

disease after the onset of symptoms. Once decisively diagnosed, the patient is subjected to a known therapy to treat that particular disease (*e.g.*, chemotherapy for cancer). As a result, medical research has focused heavily on three-step process, (i) first, diagnosis of the disease (ii) determination of the stage of the disease and (iii) developing a drug or standard of care regimen for that disease [25].

While this approach is typically the gold standard for treatment, quite a few of the lethal diseases, such as cancer, are heterogeneous in their expression [26, 27]. Therefore, in modern times individualized patient-specific treatment (*e.g.* CAR-T cell therapy), despite being expensive and out of reach for poor patients, is gaining interest [28]. Toward this end, the field of nanotheranostics has emerged, which aims to allow physicians to have a non-invasive diagnosis/prognosis in real-time. This also minimizes the chances of significant delay in case of an unforeseen adverse effect of the treatment. It should be noted that key to successful recovery from any disease is dependent on an accurate and early diagnosis. That is why ample attention needs to be paid for research in diagnostic techniques alongside developing therapeutics.

In general, a polymer-based theranostic material is comprised of at least four main features: (i) a polymer component that is biodegradable, non-toxic and non - immunogenic which carries a binding motif to capture the theranostic agent, (ii) the polymer component does not bioaccumulate and gets excreted (iii) a therapeutic agent (*i.e.*, small-molecule drug, nucleic acids, *etc.*), and (iv) an imaging agent (*i.e.*, MRI contrast agent, radionuclide, fluorophore, *etc.*). In some cases, the therapeutic component can also possess diagnostic features (*e.g.* Doxorubicin) [29]. Generally, these cargo molecules are toxic and can lead to systemic toxicity. To circumvent unwanted toxicity and to attain maximum specificity, most of these materials also incorporate targeting ligands as an additional component (*e.g.*, Hyaluronic acid that can bind to the CD44 receptor overexpressed in tumors).

Building on the progress made in developing therapeutic polymeric nanoparticles, researchers are now incorporating clinically used imaging modalities into therapeutic nanocarriers. The polymeric nanoparticles are used to deliver different imaging agents for (i) MRI (gadolinium, iron oxide, manganese oxide, ^{19}F labeled compounds) (ii) PET (1^{8}F, ^{64}Cu, ^{11}C, ^{15}O-labeled compounds) (iii) SPECT [9^{9} mTc, ^{111}In chelates), (iv) ultrasound (microbubbles, nanobubble) [24].

POLYMERS USED FOR DELIVERY OF THERANOSTICS

Different types of polymers have been used in delivery of hydrophobic drugs or genetic material.

They can be broadly classified into two major subtypes: natural and synthetic. Only a few of them are discussed in this book chapter.

NATURAL POLYMERS

Chitosan

Chitosan is a non-toxic biodegradable, cationic polysaccharide with randomly distributed α [1, 4]-linked N-acetyl-D-glucosamine and D-glucosamines. It is a good candidate for gene delivery because the amine groups present carry positive charges allowing it to form polyelectrolyte complexes with nucleic acids. The amine group present in chitosan can also be used for functionalization. Since the introduction of chitosan as a potential gene carrier, various chitosans and chitosan derivatives have been investigated, including (i) unmodified chitosans with different molecular weights and degrees of deacetylation, (ii) modified chitosan, and (iii) chitosans conjugated with receptor- specific ligands. While chitosans are biodegradable and less toxic compared to most synthetic cationic polymers, it is sparingly soluble in physiological pH. To make chitosan more soluble, modifications (quaternization by methylation or by functionalization with cationic amino acids) need to be made on the backbone of chitosan. Transfection efficiency of unmodified chitosan is often poor. Several modified chitosan derivatives have been studied over the years to improve its transfection efficiency.

To reduce the non-specific and poor cell uptake of chitosan polyplexes, researchers have investigated the conjugation of ligands, such as galactose [30] and folate [31, 32], to the backbone of chitosan. As described earlier, attachment of galactose helps polyplexes to bind to asialoglycoprotein receptors on hepatocytes [33] or the MGL receptor on antigen presenting cells [34], whereas folate conjugation is intended to target tumor cells [35].

Dextran

Dextran is a naturally occurring branched polysaccharide composed of simple repeat units of α-D-glucose [36]. Dextran is hydrophilic, water soluble and on its own unable to package hydrophobic drugs. Therefore, functional group modification is needed to introduce hydrophobic patches in dextran. Acetylation of the hydroxyl groups can make dextran amphiphilic, which can form polymeric micelles. They can also be conjugated to other polymers, such as PEG, poly(ε-caprolactone) (PCL), or polylactide (PLA), as a block copolymer to form amphiphilic micelles, which can be loaded with hydrophobic therapeutic agents [37]. As a polymeric micelle, dextran can encapsulate cargo molecules and retain them at the interior [38]. Dextrans, upon modification of its hydroxyl groups, can be used to make 3D network scaffolds to treat wound healing, tissue repair and

other uses in regenerative medicines [39].

Alginate

Alginate is a naturally occurring polymer purified from algae. It is linear polymer, consisting of irregular blocks of β-1,4-linked mannuronic acid and α- [1 - 4]-linked guluronic acid residues [40]. The most common use of alginate is to form 3D scaffolds by forming hydrogels which then serves as a carrier for cargo molecules. Alginate is biodegradable, non-toxic and nonimmunogenic. That is why alginates are used in a range of Food and Drug Administration–approved products, from food additives to wound dressings [41].

There are different ways alginate hydrogels are formed: (i) by ionic cross-linking using divalent cations like Ca^{2+} or Mg^{2+}, (ii) by covalent cross-linking utilizing the -OH and -COOH functional groups of aliginate and with crosslinkers such as glutaraldehyde, adipic acid dihydrazide, poly (ethylene glycol)-diamine, (iii) by producing hydrogels using alginate and a thermo-responsive polymer such as poly(N-isoropylacrylamide) (PNIPAM), (iv) by cross-linking ligand functionalized or cell adhesion peptide (*e.g*. arginine, glycine, aspartic acid sequence (Arg-Gly-Asp, RGD) conjugated alginates with cells and (v) free radical polymerization using functionalized alginates in presence of a photo-initiator and UV radiation [42]. It is easily functionalized owing to hydroxyl and carboxyl groups on the backbone, so its chemical and biological properties can be tuned [41].

Gelatin

Gelatin is poly-ampholyte, a mixture of proteins and peptides isolated from partial hydrolysis of animal collagen [43]. It is biocompatible, nonimmunogenic and contains both cationic and anionic groups. Gelatin polypeptide is composed of repeating triplets of alanine, glycine and proline residues, responsible for typical triple helical structure of gelatin [44]. Since it is safe and non-toxic, gelatin nanoparticles have been investigated for both nucleic acid and small-molecule drug delivery to tumors [45, 46].

There are three main ways of using gelatin for delivery of cargo molecules. (i) Carboxylate and hydroxyl groups present in gelatin are routinely used for functional group modification and make responsive version of naturally occurring gelatin [45, 46]. (ii) Modification of the isoelectric point of gelatin to tailor the loading capacity of charged drug. (iii) Tuning the molecular weight and the degree of crosslinking of gelatin to control drug release.

Poly (L-Lysine)

Poly (L-lysine) (PLL) is a polymerized version of natural amino acid L-lysine. While the positive charge containing amine functional group of PLL serves as the binding motif to bind small molecule or to condense gene products, the L-stereochemistry makes it non-immunogenic and biodegradable. Depending on the synthetic procedure both linear and branched version of PLL can be manufactured. The NH_2 functional group of PLL can also be modified to introduce ligands, cell penetrating peptides or hydrophobic patches [47].

SYNTHETIC POLYMERS

Poly (lactic-co-glycolic acid) (PLGA)

Poly (lactic-co-glycolic acid) is a copolymer of lactic acid and glycolic acid. It is probably one of the best characterized biodegradable copolymers that decomposes to non-toxic products (H_2O and $CO_{2]}$. Nanoparticles made from PLGA degrades *in vivo* through hydrolysis of the ester bonds to its monomers (lactate and glycolate) and gets excreted from body without any bioaccumulation. PLGA is an attractive delivery material because of its high stability, low toxicity, low immunogenicity and a tunable degradation rate [48]. PLGA nanoparticles are routinely used in cancer therapy using both active (ligand-receptor interaction) and passive targeting (based on enhanced permeability and retention) techniques [49].

Cyclodextrins

Cyclodextrins are water-soluble synthetic carbohydrates composed of six to eight glucose units in a ring structure with a hydrophilic exterior and hydrophobic interior. Because of the hydrophobic interior, water insoluble hydrophobic anticancer therapeutics can be loaded in cyclodextrins. The hydrophilic exterior of cyclodextrins can be functionalized with ligands, antibodies *etc* [50, 51].

Polyethyleneimine (PEI)

PEI is one of the most studied polymers in the context of gene delivery. It is manufactured in different molecular weight, linear and branched form. It is non-immunogenic and easier to scale up [52 - 55].The use of DNA-PEI polyplexes, instead of pure DNA, was demonstrated to improve the gene expression efficiency up to 100-fold [56]. This enhanced gene expression is hypothesized to be associated to the so-called “proton sponge effect” of PEI [57], that helps the polyplex to escape endosome. The proton sponge effect of PEI (and other cationic polymers) is not proven, debatable but still widely accepted.

Poly (β-amino ester)

Poly (β-amino ester) (PBAE) libraries are synthetic polymers with diverse properties, including molecular weight, charge, and hydrophobicity [58 - 63]. Just like other polymers PBAEs are cationic that bind to the cargo, surface of the cells and facilitates endosomal escape. The polymers contain ester bonds like PLGA that are hydrolyzed in physiological pH rendering them biodegradable [64].

CONCLUDING REMARKS

Compared to viral vectors, polymeric nanoparticles are still in nascent stage in terms on approval from regulatory agencies for human use. Most of the polymers are still not recognized by regulatory agencies for delivery of therapeutics. Compared to viral vectors, polymeric nanoparticles offer significant benefits such as biocompatible, biodegradable, non-toxic, non-immunogenic, well characterized and amenable for functionalization for targeted delivery. But translation of the preclinical success into clinical success is far from easy. Natural polymers are isolated from variety of sources, thus minimizing batch-to-batch variability is often challenging. Synthetic polymers are sometimes difficult to scale up to industrial scale, which in turn makes clinical trials hard to carry out. Despite all the challenges, recent success of Covid-19 vaccines from Moderna and BioNTech-Pfizer makes the future of non-viral nanoparticles bright. Once the clinical translation problem is solved, with the advancement of nanotechnology, employing polymeric nanoparticles may become a very useful approach for cancer therapy as well as treating other diseases.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 5

Magnetic Nanoparticles for Imaging, Diagnosis, and Drug-Delivery Applications

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Abstract: Magnetic Nanoparticles (MNPs) have gained interest within the research community due to their therapeutic potential in a variety of medical applications. MNPs are generally composed of a metallic core stabilized by the addition of an outer shell that can be further functionalized through the absorbance or conjugation of various targeting ligands. The magnetic properties of these nanoparticles can be utilized for imaging, localized drug delivery, and enhanced diagnostic detection. This chapter highlights the applications of MNPs to enhance magnetic resonance imaging (MRI) capabilities and improve the delivery of therapeutic agents to difficult-to-reach areas in the body. In addition, recent advances in the use of MNPs in stem cell therapy for both the tracking and monitoring of stem cell distribution in the body and improving engraftment and differentiation in stem cell therapy are discussed. Finally, examples of the incorporation of MNPs in diagnostic assays to improve rapid and real-time detection capabilities of many diseases, including cancer, cardiovascular diseases, and pathogen infections, are provided.

Keywords: Diagnostic agents, Drug delivery, Imaging agents, Magnetic Nanoparticles (MNPs), Stem cell therapy.

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INTRODUCTION

Magnetic nanoparticles (MNPs) are critical components in many biomedical applications. Although concerns regarding long-term safety remain to be addressed, MNPs are currently being explored in a variety of biomedical applications, including MRI imaging [1 - 3], bio-sensing diagnosis [1, 4], stem cell delivery, and tracking [5], gene therapy and drug delivery to treat multiple disorders including cancer, neurodegenerative diseases, HIV/AIDS, ocular diseases, and respiratory diseases [3, 6, 7]. Despite recent advances in nano-medicine using MNPs, there are many challenges to be solved in order to achieve optimal performance of these nanomaterials. The major limitations associated with MNPs include 1) development of synthesis schemes and storage and handling protocols that can be scaled-up for clinical applications while retaining necessary reproducibility in terms of size, charge, and surface properties [8, 9], 2) identification of the long-term toxicity of MNPs [9, 11], and 3) management of unexpected results due to biocompatibility and immunogenicity [9, 12].

While there are a variety of synthetic pathways and a multitude of different compositions, MNPs for medical applications all share the following common components; a magnetic core, a protective outer layer, and a surface that can be functionalized to improve targeting or biodistribution properties (Fig. **1**) [13, 14]. Magnetic nanoparticles can be further classified as either ferromagnetic, paramagnetic, antiferromagnetic, diamagnetic, or ferrimagnetic based on their core material and resulting magnetic properties. Ferromagnetic nanoparticles consist of iron, nickel, or cobalt cores and display strong magnetic moments when placed in an external magnetic field. Ferrimagnetic nanoparticles have maghemite or magnetite iron oxide core material and display similar characteristics as ferromagnetic material in the presence of an external magnetic field. Paramagnetic materials have gadolinium, magnesium, and lithium as core materials. Magnetic nanoparticles formed from paramagnetic material have weaker magnetic moments and do not sustain their magnetic properties following the removal of the magnetic field, as observed with ferromagnetic materials. The MNPs formed from diamagnetic material commonly have copper, gold, or silver cores and have the weakest magnetic moments. The strong magnetic moments of the ferromagnetic and ferrimagnetic nanoparticles make them of interest for diagnostic agents, as well as in hyperthermia-based treatment applications.

General Features of MNPs

While many magnetic core materials are available for MNP synthesis, some, such as cobalt, chromium, and alkaline earth metals, used in ferromagnetic material, are toxic and cannot be used for biomedical applications unless being coated with

a safe, non-toxic protective layer [15, 16]. Thus, despite the strong magnetic moments inherent in these MNPs, the potential toxicity of these ferromagnetic materials has limited their use as imaging agents and therapeutics for animal and human applications. In contrast, the MNPs made with magnetite and maghemite iron-oxide as core material have established safety profiles that make them more suitable for imaging and diagnostic purposes [10, 17, 18].

Regardless of the magnetic core material used, the MNPs are typically coated with biocompatible materials to prevent corrosion or the leaching of the core [11, 19]. Silane-based coatings have been particularly useful for MNPs with iron oxide core material as they readily react with the core to form a protective silane shell that can be further modified for the particular application. The ability of silane-based coatings to retain the crystallinity of the metal oxide core preserves the magnetic properties while providing an outer shell that enhances the stability of the MNPs (*i.e.*, prevents aggregation), making them ideal surface components. The utility of the silane coatings as intermediates for further surface modifications is particularly attractive. This is illustrated in the studies of Yathindranath and coworkers [20]. Using aminosilane functionalization of iron oxide nanoparticles as intermediates, Yathindranath and coworkers demonstrated the utility of silane-based surface coatings properties to accommodate changes in surface charge, through variations in amide and carboxylic acid function groups, as well as hydrophobic/hydrophilic balance, through covalent attachment of oleic acid and albumin [20]. Silane-based coatings of iron oxide nanoparticles have shown favorable safety profiles in various models [21, 22] while providing flexibility for a multitude of different surface modifications.

In addition to the silane inorganic surface modifications other coatings have also been considered. Natural coatings consisting of carbohydrates, such as dextran and starch, or proteins such as albumin and various synthetic polymers have been used as surface coatings for MNPs [23, 24]. The natural polymer surface coatings are mostly hydrophilic and require cross-linking to prevent them from disassociating in aqueous environments and to enhance their mechanical strength. In contrast, commercially available synthetic polymers used for coating purposes such as poly (ethyleneglycol) (PEG) and polyvinyl alcohol (PVA) [25, 26] are hydrophobic and display improved mechanical strength over the hydrophilic coatings. Surface modifications of MNPs are important to ensure proper attachment of biomolecules and, in the case of drug delivery applications, to control release of drug at the target site [27]. Organic linkers such as carboxylic acid and aldehyde thiol allow for the attachment of biomolecules to a variety of functional groups on the surface of the MNPs [28, 29].

There are many different synthetic procedures for the preparation of MNPs. The selection of a specific preparation technique depends on many factors, including particle size requirements, the chemical and physical properties of the active biomolecule to be carried out, and available manufacturing facilities and equipment [30, 31]. The most popular preparation methods include; wet precipitation and co-precipitation, reverse micelle mechanism, chemical vapor deposition, microemulsion, thermal decomposition, microwave-assisted combustion synthesis, carbon arc, and laser pyrolysis [30].

Physico-chemical Properties Influencing MNP Tissue Distribution

There are many physical and chemical properties of MNPs that affect their ability to reach target tissue sites. Key physicochemical properties include size and morphology of nanoparticles, as well as surface charge [32]. These physicochemical properties of the MNPs have a major influence on splenic filtration, phagocytosis, and renal clearance of MNPs. The size of MNPs greatly impacts their circulating blood half-life, which ultimately determines accumulation in target tissues. Very small particles (< 5.5 nm) can be rapidly cleared by the kidneys [33], while, large particles (> 200 nm) are more readily taken up and removed by splenic phagocytic cells [34]. Therefore, size of the MNPs factors into pharmacokinetic considerations, with too small or too large a nanoparticle resulting in rapid removal and decreased circulating half-life, and potentially, reduced accumulation in the target tissue.

In addition to particles size, the surface charge of MNPs also plays an important role in determining blood half-life. For instance, liver uptake is increased when particles and polymers have strong negative surface charges [35]. On the other hand, particles with positive charge can adsorb non-specifically to cells leading to decreased half-life in the blood [36]. From a charge perspective, neutral particles result in longer circulating half-lives compared to those MNPs with positive or negative surface charges and remain in blood circulation for longer periods of time [37].

Surface coating modifications can also be employed to enhance MNP circulation time and delay clearance from the body [38]. Mononuclear phagocytes easily opsonize uncoated MNPs and rapidly remove them from the blood. However, phagocytic removal of MNPs can be reduced by decorating the surface of the nanoparticle with organic and inorganic molecules that alter the ability to be recognized by the phagocytic cells in the blood and liver. The most common example of this is pegylation of the surface coating to avoid rapid clearance by phagocytic cells [39]. While the focus for this chapter is on the translational applications utilizing MNPs, and the results from both preclinical and clinical

studies with MNPs that support their use, the reader is referred to recent reviews that highlight additional surface modifications that are being pursued to optimize pharmacokinetic and tissue distribution properties [40, 41].

Magnetic Nanoparticles as Imaging Agents

For soft tissue, MRI provides high-resolution imaging and, when combined with the proper contrast agent, provides a powerful diagnostic tool for monitoring various pathological conditions [42, 43]. One of the most important human health-related applications of MNPs is their utilization as contrast agents to improve the diagnostic accuracy of magnetic resonance imaging (MRI). For most biomedical applications, MNPs can be categorized as either paramagnetic, ferromagnetic, or superparamagnetic particles. The composition of superparamagnetic and ferromagnetic nanoparticles is entirely different from that of paramagnetic particles. While superparamagnetic and ferromagnetic particles are composed of a magnetic core and a surface coating, paramagnetic particles are chelates of paramagnetic ions with no defined core or surface coating [44]. These composition differences between superparamagnetic and ferromagnetic particles and paramagnetic particles lead to very different profiles as MRI contrast agents.

Several studies have focused on the use of ferromagnetic nanoparticles for medical imaging applications, including magnetic resonance imaging (MRI) and Computed tomography (CT). The size of these MNPs and their strong magnetic properties make them ideal for MRI imaging. In cancer diagnosis, the nanoscale size allows for increased accumulation within the tumor tissue due to the enhanced permeability and retention (EPR) effect. The deposition of MNPs within the tumor site and the propensity for aggregation of these particles within the deep tumors enhance the MRI resolution. Furthermore, when modified with other chemical moieties, the ferromagnetic nanoparticles can be used in multimodal imaging applications. An example of this is ferromagnetic–gold nanoparticles that have proven to be useful as multimodal and dual contrast agents for MRI-CT imaging modes to produce a more precise diagnostic tool that can be used to distinguish between malignant and non-malignant tumors [45, 46].

Among the different types of MNPs, superparamagnetic iron oxide nanoparticles (SPIONs) are the most frequently used as MRI contrast agents due to their outstanding magnetic properties, biocompatibility, and biodegradability. SPIONs, by definition, display magnetic properties when in the presence of a magnetic field but are magnetically inert in the absence of an applied magnetic field. The switchable magnetic properties of SPIONs also make them attractive as drug carriers for targeted delivery to selected tissues as they can accumulate in target organs following application of an external magnetic gradient field, or as heat

conductors when in the presence of an externally applied oscillating magnetic field (thermal therapy). The composition and characteristics of the surface coating of SPIONs impact their pharmacokinetics, cellular uptake, and their ability to accumulate in target tissues. For example, macrophages efficiently take up MNPs especially those smaller than 100 nm [47, 48]. This occurs primarily through phagocytosis *via* Fc- and complement (CR)-mediated receptors. The uptake of SPIONs by Kupffer cells in the liver, red pulp and white pulp in the spleen, and histiocytes in lymph nodes have also been reported to be high and may contribute to the removal of SPIONs from the systemic circulation [49, 50]. Ultra-small SPIONs with a diameter of 5-10 nm distribute readily to lymph-node tissue, making them ideal for detecting a metastatic tumor in the lymph nodes [51]. Furthermore, due to their enhanced cellular uptake and slower clearance from the body, MNP-based contrast agents have the advantage of prolonged delineation of tumor margins [3, 51]. Clinically, SPIONs such as Ferumoxide have been used for the diagnosis of hepatocellular carcinoma (HCC) [52].

As SPIONs do not normally extravasate they are ideal agents for monitoring vascular function and have utility as MRI contrast agents for the diagnosis of several cardiovascular diseases including acute myocardial ischemic events and atherosclerosis where blood flow is altered. In a rat myocardial infarction model, SPIONs injected at a dose of 5 mg Fe/kg were used as a T2-weighted MRI contrast agent to delineate between normal and infarcted myocardium [53]. These studies suggested early detection (within 5-hrs post infarction) of ischemic, hypo-perfused heart tissue could be identified with SPION contrast agents.

MNPs as Molecular Imaging Agents

Most of the MNPs used in clinical MRI take advantage of structural differences in normal and pathological tissues (*i.e*. altered vascular permeability, increased macrophage infiltration, *etc*) to identify lesions or areas of damage. However, the high magnetic moment of MNPs compared to other conventional paramagnetic MRI contrast agents make them well-suited for use in potential molecular imaging applications. Contrast agents used as molecular imaging agents rely on molecular targets that are differentially expressed in normal *vs* disease states. In this regard, MNPs are promising contrast agents for molecular MRI as their surfaces can be functionalized with different types of ligands to target specific cellular molecules. An example of this can be found in the use of MNPs for the detection of atherosclerotic plaques. The presence of atherosclerotic plaques within coronary arteries is a leading cause of myocardial infarction and early detection of such plaques can prevent damage to the heart. Vascular cell adhesion molecule-1 (VCAM-1) is overexpressed in activated endothelial cells, macrophages and smooth muscle cells, and its expression within the vasculature can be used for the

detection of early stages of atherosclerotic plaques. In case of atherosclerotic plaque detection, it has been reported that utilizing vascular cell adhesion molecule-1-targeted MNPs greatly enhanced MRI contrast in coronary vessels of both mice and humans [54, 55].

Additional examples of SPIONs as molecular MRI contrast agents for neurodegeneration and cancer detection are in early development [56, 57]. Using a phage display library, Ansciaux *et al* identified several small peptides with nanomolar binding to beta amyloid aggregates [57]. These peptides were then conjugated to pegylated USPIONs for potential use as imaging agents for identification of beta amyloid plaques. While these studies are still ongoing, the peptide conjugated USPIONs were reported to penetrate the blood-brain barrier (BBB), and preferentially bind to amyloid deposits [57]. Importantly, initial studies indicated these MNPs were non-toxic with limited long-term deposition in tissue outside of the brain [57].

An additional example in the area of molecular contrast agents for cancer imaging and diagnosis are the studies by Bates and colleagues. Prostate-specific membrane antigen (PSMA) is a marker implicated in prostate tumor progression and metastasis. Bates and colleagues conjugated a monoclonal antibody to PSMA to a commercially available SPION [58]. The resulting antibody-SPION complex displayed targeted uptake by PSMA expressing prostate cancer cells. The favourable T2 relaxation of the antibody SPION was ideal for use as an MRI contrast agent for detection of prostate cancer.

To further improve the quality of MNPs as MRI contrast imaging agents, other modifications in the surface structure of MNPs have been examined. The addition of fluorescent or radioactive labels to MNPs allow dual imaging applications using MRI, optical or positron emission tomography (PET) imaging modalities [59, 60]. In this regard, the ability to transfect cells with fluorescent proteins have provided a method for tracking the effectiveness of MNP as gene delivery agents. An example of this is the work of Uthoman and colleagues with chitosan coated SPIONs for gene delivery to the liver. After transfection of these complexes with plasmid green fluorescent protein (GFP) into hepatocytes, gene expression was strongly observed in the cytoplasm of hepatocytes [61]. Thus, this particular MNP allowed for the potential monitoring of MNPs to target tissue with MRI and detection of the delivered MNP payload with fluorescence imaging techniques. Conjugating SPIONs with radioactive tracers has also been utilized to combine MRI with other imaging modalities such as positron emission tomography to improve medical diagnosis [62].

MAGNETIC NANOPARTICLES (MNPS) AS DIAGNOSTIC AGENTS

Recently, several studies have highlighted the importance of superparamagnetic nanomaterials as powerful diagnostic and prognostic biosensors to robustly detect low abundance, disease-specific, biomolecules in biological fluids (*i.e*. blood and urine). The use of these MNPs in diagnosis increases the accuracy of detecting many diseases at early stages. The ability to readily functionalize MNPs with different types of ligands including antibodies, peptides, and sugars, makes these particles useful as diagnostic agents [63 - 66].

Of particular interest from a diagnostic standpoint is the coupling of MNPs to enzymes. These nanomaterials called nanozymes, have an intrinsic enzymatic activity, including peroxidase, catalase, superoxide dismutase that can be utilized for detection-based assays. Kim *et al* developed a glucose biosensor made of poly-γ-glutamic acid/chitosan hydrogel nanoparticles (PGA/CS NPs) with glucose oxidase (GOx) conjugated magnetic nanoparticles (MNPs) entrapped within the hydrogel matrix [67]. In the presence of glucose, the activated GOx-MNPs entrapped in the hydrogel generated H_2O_2 that subsequently triggered the conversion of chromogenic substrate, 2,2'-azino-bis(3-ethylbenzothiazoli-e-6-sulphonic acid) diammonium salt (ABTS) [67]. Farshchi *et al* prepared novel electroconductive Fe_3O_4 MNPs conjugated with cysteamine functionalized gold nanoparticles for use as a biosensor for prostate cancer diagnosis [58]. A biotinilated-anti-prostate-specific antigen (PSA) antibody was conjugated with iron oxide magnetic nanoparticles (Fe_3O_4) and casted on the surface of a glassy carbon electrode (GCE). An encapsulated secondary antibody (HRP-Ab2) on gold nanoparticles and caped by cysteamine was then immobilized onto the surface of GCE modified electrode. The engineered interface was used as substrate to amplify and detect the electrochemical signal of antibody-antigen binding specific for prostate cancer in various biofluids [58].

The use of MNPs for toxicological screening was demonstrated by Wang *et al* to create a novel magnetic biosensor to quantitatively detect the presence of food pollutants such as botulinum neurotoxin type A (BoNT/A) and staphylococcal enterotoxin B (SEB). In this particular application, magnetic quantum dot NPs (MagQD NPs) were produced by attaching carboxylated QDs onto the surface of polyethyleneimine-modified Fe_3O_4-MNPs and applied in a lateral flow assay (LFA) [68].

The COVID-19 pandemic that has caused worldwide disruption of economies and impacted the lives of many people has seen an unprecedented need for the development of robust and sensitive diagnostic methods for detecting the virus. In this regard, MNPs have been incorporated into COVID-19 diagnostic tests. Torres

et al reported an optimized approach for preparation of MNPs used for high quality RNA extraction required for real time RT-PCR detection of SARS-CoV-2 [69]. The approach used 3-aminopropyl triethoxysilane (APTES) to coat magnetite nanoparticles. These magnetite nanoparticles were used as intermediates and subjected to further diacrylate-amine polymerization and coating with poly (amino-ester) to form the final MNP product (Poly-NH2-MNP) [69]. The resulting cationic MNP binds RNA that is then readily isolated with magnetic separation techniques. Developing novel MNPs for diagnostic purposes can help in establishing simplified and accurate diagnostic tests and procedures for early, rapid and sensitive detection of many serious illnesses.

MAGNETIC NANOPARTICLES (MNPS) AND STEM CELLS

Therapeutic application of stem cells in regenerative medicine holds great promise for numerous debilitating disorders [70]. Both preclinical and clinical studies support the potential effectiveness of stem cell therapy in mitigating the symptoms of many serious illnesses and initiating repair of damaged organs [71 - 73]. Stem cells are of interest as therapeutic agents owing to their inherent capacity for self-renewal and multi-lineage differentiation that are essential for regeneration of damaged tissue [74]. Different types of stem cells have been studied in the field of regenerative medicine including, mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and endothelial progenitor cells. Despite remarkable advancements in the area of stem cell therapy, challenges such as poor homing and retention at the injury site after transplantation, immune rejection, undesired pre-transplantation differentiation and poor survival of the transplanted stem cells within the local tissue microenvironment limit effectiveness. In this regard, MNPs have found applications for enhancing the labeling and tracking of stem cells within the body and promoting stem cell survival.

Use of MNPs for Labeling and Tracking Stem Cells

Superparamagnetic iron oxide nanoparticles (SPIONs) are small crystals of iron oxide (either maghemite γ-Fe_2O_3 or magnetite Fe_3O_4) that have been surface modified to attain colloidal stability in an aqueous medium [75]. The superparamagnetic properties allow the SPIONs to be particularly useful as MRI contrast agents. Clinically, Ferumoxide (Feridex®), Ferumoxtran, (Combidex®) and Ferumoxytol (Feraheme®) have been used as MRI contrast agents to image macrophages, lymph nodes, liver and kidney in a variety of different pathological conditions including cancer, stroke and multiple sclerosis [75 - 78]. On the pre-clinical side, efforts to functionalize SPIONs with surface modifications that

target specific cells and/or pathological targets have received a great deal of attention [31, 79].

While SPIONs are widely used for imaging purposes, using SPIONs for stem cell labeling and tracking is a relatively new application. Recently, ferumoxytol (Feraheme®, AMAG Pharmaceuticals), an ultrasmall SPION used clinically as an MRI contrast agent and as an iron supplement for treating iron deficiency in patients with chronic kidney disease [80], has also been used to label human mesenchymal stem cells (MSCs), human embryonic kidney 293 cells, and induced pluripotent stem cells [81]. Labeling of the MSCs takes advantage of the inherent phagocytic properties of these cells. Ferumoxytol colloidal particle size is less than 50 nm and can be phagocytized efficiently by the MSCs. Skelton *et al* labeled cardiac progenitor cells derived from human embryonic stem cells with ferumoxytol and then transplanted these labeled cells directly into the myocardium of the left ventricular wall of uninjured pig hearts [82]. The tissue disposition of the ferumoxytol labeled cells were then imaged and tracked by MRI for up to 40 days [82]. Using this method Skelton and colleagues were able to show that the journey and the fate of ferumoxytol labeled cells can be effectively imaged and tracked long-term using MRI.

FeraTrack™ is another SPION (direct contrast nanoparticle) that has been used with MRI as a cell tracking agent [83, 84]. These dextran coated SPIONs have a positive surface charge making them cell penetrable through a vesicular endocytosis route [83]. As no additional exposure to potentially toxic transfection reagents were necessary for labeling the cells, FeraTrack™ has gained utility as a biocompatible MRI contrast agent for cell tracking [85]. Preclinical studies demonstrate the utility of FeraTrack for monitoring MSCs at the site of cellular injury. This is elegantly illustrated by the studies of Mesentier-Louro *et al* in which FeraTrack was used to track bone marrow derived MSCs at sites of injury in a rodent model of optical nerve injury [86]. These studies showed that following intravitreous injection, FeraTrack™ labeled MSCs migrated to the site of optical nerve injury and remained there for up 18 weeks [86]. As the FeraTrack labeled MSCs were imaged using MRI these studies provided a non-invasive method for monitoring regenerative cells within the site of nerve damage.

The labeling of the stem cells with SPIONs can be further improved through surface modifications and exposure to external magnetic fields. Studies reported that the incorporation of cationic compounds such as poly-l-lysine and protamine onto the surface coating of SPIONs can enhance labeling by promoting interactions with the negatively charged cell surface [87, 88]. Further, using an external pulsed magnetic field (PMF) also improved the interaction and subsequent uptake of SPIONs in mesenchymal stem cells (MSCs) [89 - 91]. Using

an external PMF reportedly opened channels in the cell membrane and increased the uptake of SPIONs by MSCs [89].

Ngen *et al* reported a method using dual-contrast agents, combining SPIONs and gadolinium chelate to monitor stem cell delivery and homing by MRI [92]. This technique takes advantage of the slow diffusing SPIONS and the fast diffusing gadolinium based chelates [92]. The slow diffusing SPIONs generate negative contrast, resulting in loss of signal, while gadolinium chelates generate positive contrast, represented by an increase in signal gain upon its presence in tissues. Gadolinium dependent positive contrast is quenched in live cells by SPIONs, whereas, contrast enhancement is found in the vicinity of dead cells due to the release of gadolinium based chelates [92].

Extracellular vesicles (EVs) are secreted lipid bilayered vesicles containing enzymes, nucleic acids and lipoproteins that are involved in intercellular communication. Extracellular vesicles play a vital role in various diseases including cancers [93, 94]. Therefore, to assess the role of EVs, visualization and investigation of their spatiotemporal properties is crucial. To aid in such studies, SPIONs were employed to facilitate EV imaging. Dabrowska *et al* labeled the EVs derived from MSCs using the fluorescent lipophilic stain PKH26 and SPION nanoparticles conjugated with rhodamine (Molday ION Rhodamine B™) [95]. This labeling combination was biocompatible with EVs and allowed the imaging of EVs using TEM and MRI.

Guidance and Homing of Stem Cells

The success of stem cell therapy is dependent on the ability to precisely deliver and retain regenerative cells at the target site. In order to monitor the reach of stem cell populations, tracking of cells is essential. In the recent years, paramagnetic nanoparticles were used for stem cell tracking [96, 97] and to control the spatial position of stem cells *in vivo* [98]. In a recent study, Braniste *et al* synthesized semiconductor nanoparticles by growing nanometer scale GaN thin layers on a zinc ferrite core ($ZnFe_2O_4$) [99]. Interestingly, incubating mesenchymal stem cells with these low density (10 mg/ml) semiconductor nanoparticles was enough to redistribute and rearrange them according to the remote magnetic field intensity, which was important for long term tracking and monitoring of the injected cells [99].

Silva *et al* evaluated the biocompatibility of gold and maghemite nanoparticles functionalized with 2,3-dimercaptosuccinic acid (DMSA) (Au-DMSA and γ-Fe_2O_3-DMSA) with human mesenchymal stem cells [100]. They found that while both γ-Fe_2O_3-DMSA and Au-DMSA were biocompatible with mesenchymal stem cells, they were unsuited for tracking these cells *in vivo* by standard

computed microtomography [100]. The following possibilities were considered for the loss of Au-DMSA signals in microtomography: (a) Au-DMSA based contrast in MSCs was not enough to distinguish MSCs from the surrounding connective tissue and (b) fewer MSCs were labeled through intranasal route leading to loss of signals.

Panseri *et al* tested the effect of iron-doped hydroxyapatite nanoparticles (FeHA NPs) on the viability of MSCs. They found higher cell viability in-respect to commercial SPION formulations [101]. The improved cell viability observed with MSCs treated with FeHA NPs were attributed in part to the more rapid degradation and lower resulting intracellular iron concentrations with the FeHA NPs compared to SPIONs. Additionally, the intrinsic properties of FeHA NPs could be utilized as an effective magnetic carrier for many therapeutic products including drugs, growth factors, and miRNA [101].

A recent study by Krasteva *et al* used a modified method to label and count CD34+ stem cells. In this study, Krasteva *et al* prepared an immobilized conjugate of anti-CD34+ monoclonal antibody-dR110 and free conjugate anti-CD45+ monoclonal antibody-ATTO620 onto a functionalized MNPs [102]. The functional MNPs were synthesized by using thermal co-precipitation of Fe^{2+} and Fe^{3+} salts in aqueous solution and then modified with 3-aminopropy-ltriethoxysilane for formation of amino groups onto the magnetic surface. This method of labeling yielded a more accurate counting of the stem cells with little toxicity as demonstrated by the high viability (98.5 to 99.6%) of labeled cells [102]. Ledda *et al* examined the biocompatibility and the biosafety of ultrafine silica-coated superparamagnetic iron oxide fluorescent nanoparticles (sub-5 SIO-Fl) by injecting this MNP into a mouse model [103]. The biodistribution and toxicity profile was investigated for a 7-week period. In this study, Ledda *et al* reported no apparent toxicity with a wide body distribution profile of sub-5 SIO-Fl. This study displayed encouraging outcomes for the possibility of using sub-5 SIO-Fl for labeling and diagnostic purposes [103]. Similarly, Ahmad *et al* reported a high safety profile of triple polymer-coated magnetic nanoparticles (MNP-AC-G2-pPEG) following intravenous administration in Swiss albino mice [104]. Moayeri *et al* used a poly-L-lysine hydrobromide coated superpara-magnetic iron oxide nanoparticles (SPIONs) to label adipose-derived stem cells (ADSC- SPION/PLL) [105]. After loading, these ADSC-SPION/PLL stem cells were then transplanted in the medial forebrain bundle into a rat model of Parkinson Disease (PD), and the resulting migration and homing of the stem cells to lesion sites were examined. They found that application of external magnetic field by placing a magnet on the top of rat skull for 2 weeks increased the number of AD-MSCs labeled with SPIONs in the damaged site of substantia nigra [105].

Improving Differentiation and Stem Cell Survival

While the superparamagnetic properties of MNPs make them ideal agents for labeling and tracking of stem cells within the body, investigations have demonstrated these same properties also provide advantages for stem cell survival and differentiation. MNPs can be used for improving the cryopreservation of stem cells. Ito *et al* demonstrated successfully the possibility of cryopreserving large volumes (20 ml) of human induced pluripotent stem cells (hiPSC) using a "nano-warming" method that was based on magnetic nanoparticles coated with mesoporous silica and a magnetic field [106]. In addition to improving the storage of stem cells, the stem cells MNP combination also potentiated the proliferation, differentiation and repair abilities of the stem cells [106].

Naseroleslami *et al* investigated the protective effects of human amniotic membrane-derived mesenchymal stem cells (hAMSCs) labeled with (SPIONs) in a rat model of isoproterenol (ISO)-induced myocardial injury [107]. This study demonstrated improved cardiac repair abilities of SPION-labeled hAMSCs in the presence of magnetic field through suppression of NF-κB/MAPK dependent inflammation [107]. Wang *et al* fabricated a magnetic lanthanum-doped HA/CS scaffold to facilitate stem cell retention at the site of injury (MLaHA/CS) [108]. These studies reported significantly enhanced recruitment of endogenous MSCs that facilitated regeneration of new bone matrix after implanting the MLaHA/CS scaffolds into rat calvarial defects [108]. Congruently, Zhang *et al* modified Fe_3O_4 MNPs by the addition of graphene oxide (GO) to generate Fe_3O_4@GO magnetic nanocomposites (MNCs) [109]. The Fe_3O_4@GO MNCs were able to attenuate the cell damage caused by oxidative stress and improved the osteogenic differentiation of MSCs through application of a magnetic field and bone morphogenetic protein-2 (BMP2) [109].

As cell behavior and stem cell fate can be influenced by magnetic nanoparticles, incorporation into various cell scaffold material has been examined to improve the bioactivity of the scaffolds intended for various tissue regeneration. Nazari *et al* constructed electrospun magnetic scaffolds using silk fibroin (SF) and incorporating casein modified SPIONs (a surface modified SPIONs) directly into the electrospinning solution [110]. The resulting casein-modified SF-SPIONs were found to possess improved water solubility, biocompatibility and stability. The prepared scaffold was then used as a cellular matrix for culturing mouse embryonic cardiac cells (ECCs). Compared to conventional culture plastic, the SF-SPION nanofiber scaffolding for culturing ECCs resulted in increased expression of cardiac functional genes, including GATA-4 and Cardiac Troponin T that are correlated with better cardiac differentiation abilities without the influence of any external magnetic field [110]. These studies exemplify how

SPIONs have been successfully employed to control stem cell homing and differentiation. The external magnetic field can dramatically influence the direction of stem cells differentiation. Labusca *et al* studied the effect of duration, intensity and frequency of magnetic field on the differentiation abilities of adipose derived stem cells (ADSCs) labeled with MNPs [111]. Surprisingly, these investigators found that using a low intensity magnetic field (0.5 MT) intermittently for short periods (2 days) favored adipogenesis, while applying intermittent high intensity magnetic field (21.6 MT) for short periods (2 days) or continuous low intensity magnetic field (0.5 MT) for longer periods (7 days) increased osteogenesis [111]. Additional studies by Wang *et al* injected SPION-labeled ADSCs in a rat model of stress urinary incontinence (SUI). The magnetically labeled stem cells achieved a high retention rate post transplantation and facilitated the restoration of sphincter structure [112]. Together these findings with the MNP labeled stem cells highlights the advantages that incorporation of MNPs in stem cell therapy may have for improving successful outcomes. Future studies should be planned to investigate and confirm the long-term efficacy and safety of these MNPs labeled stem cells, beside investing in conducting clinical trials to reveal the translational possibilities of these MNPs–labeled stem cells.

MAGNETIC NANOPARTICLES FOR DRUG DELIVERY AND GENE THERAPY

Magnetic nanoparticles (MNPs) are also of great interest for drug delivery and gene therapy research fields [113]. MNPs formulations for drug delivery have several advantages. One advantage is that the magnetic core of the nanoparticles allows for potential site selective delivery through application of an external magnetic field [114]. The importance of a magnetically driven delivery system is the ability to deliver the bio-therapeutic materials directly to the specific site within the target organ while minimizing accumulation in other tissues [115]. The magnetic properties are dependent on the core material. For magnetic driven drug delivery applications, the most frequently used core materials include maghemite (γ-Fe2O3) and magnetite (Fe_3O_4) [17]. Coating the core material with polymers such as polyethylene glycol or dextran provided a surface for covalent attachment of small molecules drugs or biological therapeutic agents [116]. Encapsulation of drug and core material within solid lipid or liposomal preparations was also possible [117]. For magnetically driven drug delivery applications, the size and shape of the core metal material and the type of surface coating applied can have dramatic effects on the mobility of MNPs in a magnetic field [118].

MNPs in Respiratory Drug Delivery

Magnetized aerosols, referred to as nanomagnetosols, are formulated specifically

for inhaler based applications by combining MNPs with a soluble drug, siRNA or gene product and directing passage through the airway through the use of an external magnetic gradient field [119, 120]. Magnetic aerosols are used to enhance drug deliver to specific targets in the respiratory system while minimizing side effects [6, 121]. This is particularly advantageous for chemotherapeutic applications for the treatment of lung cancer. Hasenpusch *et al*. used two different nanomagnetosole inhalation formulations to demonstrate enhanced pulmonary delivery in a murine model [122]. First, nanomagnetosoles containing SPIONs labeled with fluorescein were used to track the SPIONs within the airway. A second nanomagnetosole formulation containing SPIONs adsorbed with polyethylenimine-plasmide DNA (PEI-pDNA) complexes were also examined. The authors showed that in the presence of an externally applied magnetic field, the magnetized aerosols demonstrated enhanced pulmonary delivery and gene transfection efficiency in the deeper airways [122]. These are promising pre-clinical findings that await further development and translation to the clinic.

MNPs in Ocular Drug Delivery

MNPs have also been explored for drug delivery to specific targets in the eye such as the retina and retinal pigment epithelium. The anatomy and physiology of the eye make efficient drug delivery to these sites very challenging [123, 124]. This is especially the case for biologicals and gene-based therapeutics. Using MNPs as ocular gene delivery systems have been investigated in animal models. Nerve growth factors and brain derived neurotrophic factor have been efficiently delivered intraocularly to zebrafish by using MNPs to prevent oxidative retinal damage. Giannaccini *et al*. showed that MNPs can be injected intraocularly in xenopus and zebrafish and rapidly enter into and localize within the retinal pigment epithelium without causing tissue damage [124]. Amato *et al*. recently showed the intraocular delivery of the somatostatin analog octreotide with MNPs (MNP-OCT) for diabetic retinopathy therapy. Octreotide inhibited the overexpression of vascular endothelial growth factor and apoptotic cell death associated with diabetic retinopathy [125, 126]. In these studies, the MNP-OCT were injected intraocularly and localized mainly in the retinal pigment epithelium in mouse eye. The localization of MNP-OCT enhanced the octreotide bioavailability and avoided the need for frequent octreotide intraocular injections. As repeated intraocular injections of octreotide can cause ocular hypertension, retinal detachment, and cataracts, the use of MNP-OCT for intraocular injections may reduce the adverse effects from repeated octreotide intravitreal injections [126].

MNPs in Neurological Disorders

MNPs have shown great promise in treatment of neurological illnesses [127]. Applying an external magnetic field helps to control the movement of MNPs and increase drug bioavailability in the brain. Preclinical studies using an *in vitro* blood-brain barrier (BBB) model showed that MNPs increased trans-endothelial permeability of the drugs [128]. Additional studies by Sun and colleagues demonstrated that transient disruption of the brain endothelial cells combined with an external magnetic field increased the passage of MNPs in the cell culture model of the BBB [129]. The process, termed Magnetic Field Enhanced Convective Diffusion (MFECD) was recently used to demonstrate improved delivery of anticancer agents salinomycin and doxorubicin across *in vitro* BBB models [130, 131].

Improved brain penetration of drugs using MNPs has also been observed in pre-clinical animal studies [132, 133]. MNPs can be embedded with liposomes (magnetoliposomes) to protect the loaded drugs from uptake by reticuloendothelial system, increase drug concentration in the brain, and reduce biological degradation [134]. MNPs have been used to improve the delivery of anti-retroviral drugs such as 3′-azido-3′ -deoxythymidine-5′ -triphosphate (AZTTP) into the BBB *in vitro* models. Using the AZTTP-MNPs and AZTTP-magnetoliposome formulations in the presence of an external magnetic field showed significantly increased AZTTP deliver through the BBB compared to free AZTTP [134, 135]. In addition, MNPs enhanced synaptic plasticity following HIV infection. As illicit drugs can accelerate synaptic plasticity damage during HIV infection, combining anti-morphine drugs such as D-Pen-Cys-Tyr--Trp-Orn-Thr-Pen-Thr-NH2 (CTOP) with MNPs facilitated delivery through the BBB and protected the mice from neuronal plasticity dysregulation [128].

Nerve growth factor (NGF) plays a fundamental role in nerve cell regeneration and repair. Several delivery platforms incorporating MNPs have been used to provide NGF to stimulate neurite outgrowth [136 - 138]. Marcus *et al*. have used MNPs to deliver nerve growth factor (NGF) *in vitro* to stimulate PC12 cell differentiation and outgrowth under an external magnetic field control [136]. Conjugation of iron oxide nanoparticles and NGF resulted in improved stability of NGF and significantly promoted neurite outgrowth in PC12 cells compared to free NGF [136]. Similar results were also reported using poly-L-Lactic acid (PLLA) microspheres incorporating NGF and MNPs [137, 138].

Application of MNPs in neurodegenerative diseases is of great interest. Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by chronic progressive degeneration of nerve cells.

Conventional drugs have reduced ability to cross the BBB, resulting in decreased bioavailability in the brain and poor therapeutic responses. The main characteristics of AD are extracellular aggregation of amyloid-β (Aβ) and intracellular neurofibrillary tangles associated with tau protein [139]. Therefore, various treatments directed at reducing Aβ and tau protein accumulation and production in the brain have been proposed. Targeting Aβ with specific monoclonal Aβ antibody in the brain by using MNPs helps to inhibit Aβ aggregation and improve AD symptoms. Previous studies reported that SPIONs coated by PEG-NH2, when placed under an external magnetic field, reduced the formation of Aβ aggregates [140]. MNPs have also been used in neurodegenerative tauopathy therapies to inhibit microglial activation. Glat *et al* showed that microglial activity could be significantly inhibited by systemic administration of γ- Fe_2O_3 iron oxide nanoparticles conjugated with fibrin γ377–395 peptide in an *in vivo* tau-mutant mice model, compared to peptide treatment alone [141]. Conjugating the peptide with MNPs lead to increase peptide stabilization and potentially inhibited the microglial activation [141].

MNPs are also considered a promising treatment for PD. Aggregation of phosphor-α-synuclein molecules in dopaminergic neurons leads to damage the dopaminergic neurons and decreased dopamine secretion resulting in movement disorders [142]. Targeting α-synuclein in neurons inhibits α-synuclein expression and prevents cell toxicity and apoptosis. Niue *et al*. delivered short hairpin RNA (shRNA) using MNPs to inhibit α-synuclein expression. shRNA was absorbed on the magnetic Fe_3O_4 nanoparticles coated with oleic acid then delivered to the neurons where suppression of α-synuclein expression was observed [143]. Magnetically guided drug or gene delivery with MNPs is a very promising method to improve drug delivery and gene therapy in the brain.

MNPs in Gene Therapy

MNP-based gene delivery systems have been explored in various diseases including cancer, autoimmune diseases, diabetes, and viral infection [144]. Both viral and non-viral vectors have been complexed with MNPs and used for various *in vitro* and *in vivo* gene therapy applications. The advantage of MNPs that use non-viral methods for cellular delivery of genetic material is that these systems have less immunogenic effects and are less toxic than MNPs that incorporate viral vectors. However, MNPs using viral delivery carriers are more effective for DNA delivery [145, 146]. An additional advantage of MNPs for delivery of both genes and siRNA centers is the ability to improve transfection efficiency by the application of an external magnetic field [147]. This process called, magnetofection, increases gene transfection through the improved cellular uptake of the MNPs in the presence of an external magnetic field.

In vivo applications of improved gene delivery with MNPs and magnetofection have also been reported. Studies demonstrated that following intravenous injection of MNPs with a gene construct, an external magnetic field could be applied to transfer the MNPs to specific tissue [7]. The MNPs can also increase gene stability and protect the gene from nuclease degradation, which facilitates more effective gene delivery [148]. Non-viral MNP formulations were used to activate the apoptosis pathway in breast cancer cells and to inhibit tumor growth [149]. In an interesting combinatory approach, investigators transfected vascular endothelial cells *in vitro* using a MNP-lentiviral vector conjugate and then used the transfected cells for vascular repair [150]. Vosen *et al*. showed that *in vitro* transfection of vascular endothelial cells with MNP-endothelial nitric oxide synthase (eNOS) lentiviral vector complexes increased cellular nitric oxide levels [150]. The eNOS expressing cells were then injected intravenously into the mouse. The transfected eNOS expressing endothelial cells were directed to the damaged vasculature using an external magnetic field where the eNOS expression in endothelial cells was able to initiate vascular repair and improve vascular function [150]. As dysfunction of endothelial cells within the atrial vasculature is a leading cause of atherosclerotic cardiovascular disease, this repair approach may have clinical importance.

MNPs in Cancer Therapy

One of the major areas of drug delivery using MNPs is cancer treatment [14]. Most conventional anticancer drugs are non-targeted and have serious adverse effects on healthy cells. MNPs can be used to overcome these disadvantages with anticancer drugs [151]. Treatment of tumors such as intramedullary spinal cord tumors (IMSCTs) is very challenging [152]. Treatment with conventional chemotherapeutic drugs suffers from reduced ability to penetrate the blood-spinal fluid barrier and systemic toxicity [153]. Kheirkhah *et al*. reported a novel treatment of IMSCTs using a MNP-doxorubicin complex together with application of an external static magnetic field to concentrate chemotherapy to the tumor site. In these proof-of-principle studies, an IMSCT was induced in a rat model and following initial tumor growth, doxorubicin-MNPs were injected intrathecally at L3/L4 vertebral level near the tumor site. Implantation of a neodymium magnet sub-dermally overlying the laminectomy site provided a static magnetic field at the tumor site that held the MNPs near the tumor site. The rats were sacrificed after one week of doxorubicin-MNPs injection, and cellular uptake and intracellular localization of doxorubicin-MNPs were evaluated using confocal fluorescence microscopy. The authors found this treatment approach increased doxorubicin localization within the tumor site and induced cell apoptosis in tumor cells [154].

A growing area of interest in cancer treatment is the use of MNPs for thermal therapy. In this approach, MNPs are subjected to an alternating magnetic field. The heat generated by the movement of the MNPs in the oscillating magnetic field creates localized hyperthermic conditions and causes tumor cell death. Cancerous cells are thermally sensitive, and magnetic hyperthermia, either alone or in combination with chemotherapy, can result in decreased viability of cancer cells. Studies by Sadhukha *et al*. reported that cancer stem cell (CSC) growth could be inhibited through magnetic hyperthermia produced by subjecting superparamagnetic iron oxide nanoparticles (SPIONs) to an alternating magnetic field [155]. As CSCs are difficult to treat with conventional therapy and responsible for relapse, methods for effectively treating these cells hold the key to cancer treatment. Interestingly, the cytotoxic effects observed with magnetic hyperthermia in CSCs were greater in magnitude than that produced by conventional hyperthermia treatment, suggesting activation of additional cellular pathways, such as reactive oxygen species, following hyperthermia with MNPs [155]. As the amount of heat produced can be influenced by the size and the shape of the MNPs and the frequency of the alternating magnetic field [156], hyperthermia applications for MNPs remain of considerable interest [157].

Despite many different MNP formulations examined in preclinical hyperthermia applications, clinically, NanoTherm® is the only nanoparticle thermal therapy formulation that has been approved for use in cancer therapy. NanoTherm® is a colloidal suspension of aminosilane-coated SPIONs, that is administered percutaneously into the target tumor tissue. In 2010, the European Medicines Agency (EMA) approved the use of NanoTherm® to treat recurrent glioblastoma multiforme (GBM). Clinical trials in glioblastoma patients showed both tolerability as well as significant improvements in re-occurring tumors following treatment with NanoTherm® [158]. Additional trials in prostate cancer reported transperineal injection of the aminosilane-coated SPIONs, and magnetic hyperthermia treatment was well-tolerated and resulted in reduced prostate-specific antigen levels in 8 out of 10 patients [159]. Although clinical trials are limited, the positive responses observed with NanoTherm® have increased interest in this area [160, 161].

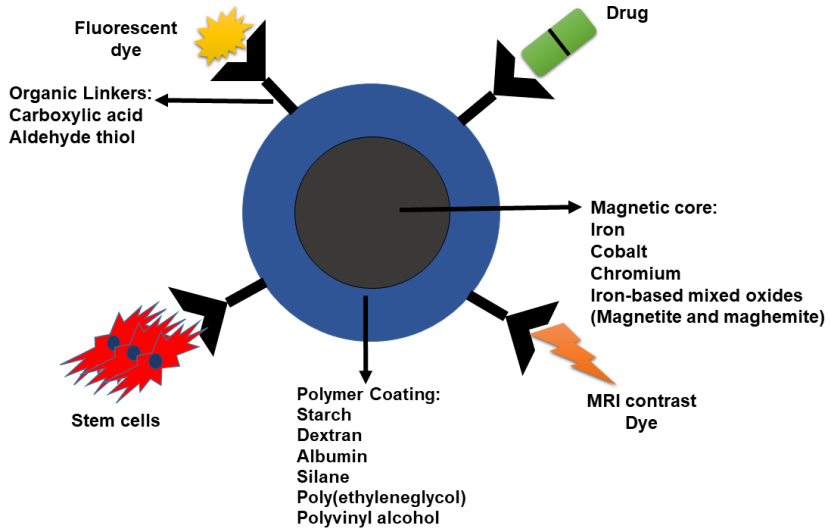

Fig. (1). Schematic representation of the basic structural components of MNPs used for clinical applications.

MNPs are also used as intrinsic anticancer agents for cancer immunotherapy. Fe_3O_4-ZnO-NPs were used in an animal model to increase antigen uptake by dendritic cells without the need to use transfection agents. Dendritic cell immunotherapy is based on the activation of antigen-specific cytotoxic T cells to reduce the growth of tumor. Cho *et al*. showed that Fe_3O_4-ZnO-NPs helped to deliver tumor antigens efficiently into dendritic cells, which then activated cytotoxic T cells and inhibited tumor growth [162]. Injecting Mitoxantrone-SPIONs into the tumor in rabbits in the presence of an externally applied magnetic field inhibited tumor growth without adverse effects [163]. Also, MNPs can control the release of dual drugs under magnetic hyperthermia. Hu *et al*. designed SPIONs coated with heat-sensitive polyvinyl alcohol combined with two anticancer drugs (doxorubicin and paclitaxel). The authors showed that the release of both drugs from the MNPs could be controlled by the application of an external magnetic field and resulted in enhanced tumor regression [164]. Sato *et al*. showed that the ability of cisplatin to enhance apoptosis of cancer cells *in vitro* was enhanced by magnetic hyperthermia generated with ferucarbotran in altering the magnetic field [165].

CONCLUSION

There is an emerging role for MNP in the imaging, diagnostic, and drug delivery fields. The intrinsic properties of the MNPs, namely their ability to be attracted to or retained at specific sites through the use of externally applied magnetic fields, provide advantages for targeted delivery. Furthermore, their high magnetic moments, especially for the SPIONs, make them ideal for MRI-based imaging

and diagnostics. The ability to modify the surface properties and shape of the MNPs to fit the specific application make these nanoparticles highly adaptable and useful for future biomedical applications.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 6

Aptamers in Theranostic Bionanomaterials

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Abstract: Theranostic nanomaterials hold the potential to revolutionize future disease management. Recent progress in nanomaterials technology and aptamer-base--targeting molecules have promoted efficient theranostics models. Aptamers are unique three-dimensional structures consisting of oligonucleotide (25-80 nt) polymers. They are comparable to monoclonal antibodies in their receptor-driven binding efficacy toward specific target receptors and binding ability to specific target molecules with high affinity and specificity. Aptamers have several other advantages, including prolonged shelf life, little or no variation from batch to batch, and ease of chemical modifications for enhanced stability and targeting capacity. Owing to the advantages mentioned above, aptamers are attracting great attention in diverse applications ranging from therapy, drug delivery, diagnosis, and functional genomics as well as biosensing. Herein, the aim is to give an overview of aptamers, highlight the opportunities of their application as means of effective therapeutic tools as well as functionalize them as potential diagnostic probes. Furthermore, the diverse modifications of aptamers for theranostic purposes, including therapeutic agents and targeted delivery nanomaterials, are comprehensively summarized.

Keywords: Aptamers, Bionanomaterials, Nanomaterials, Theranostics.

INTRODUCTION

Over the last decade, theranostic bionanomaterials have emerged as an invaluable tool in personalized medicine [1]. The term theranostics refers to the combination of molecular imaging and therapy. Indeed, some bionanomaterials per se have

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very interesting therapeutic and diagnostic characteristics [2]. Recent progress in nanomaterials technology and aptamer-based targeting molecules have provoked efficient theranostics models.

Aptamers are functional single-stranded DNA or RNA oligonucleotides ranging in size from 5 to 30 KDa that can fold into unique three-dimensional structures, providing an ability to bind their targets with high affinity and selectivity [3, 4]. Aptamers were first described in 1990 by two independent groups. Tuerk and Gold worked on translational regulation of the DNA polymerase gene to find nucleic acid ligands that interact with T4 DNA polymerase and repress its activity in an attempt to get a better understanding of the recognition site. By the end of their experiment, they were able to produce a high-affinity RNA ligand through a novel protocol termed SELEX (Systematic Evolution of Ligands by EXponential enrichment). In the meantime, Ellington and Szostak isolated specific RNA ligands capable of binding organic dyes, and they, in turn, called these ligands as Aptamers [5, 6]. Since the discovery of aptamers, several major advances have been witnessed in the aptamers field. A main progress was achieved by introducing chemical modifications of aptamers, thereby enhancing stability and binding properties [7]. Moreover, aptamers were selected for a wide range of targets, including ions, small chemical molecules, peptides, proteins, infected cells, pathogens, and even cancer cells. In the following years, aptamers found their way into several applications involving diagnostics, therapeutics, regulation of gene expression, high throughput screening, and targeted drug delivery. In 2004, Macugen® (pegaptanib sodium) was announced as the first aptamer FDA approved for the treatment of age macular degeneration disease (AMD).

Theranostics bionanomaterials have been created great growth avenues for the development of precision medicine. Various aptamer-dependent systems were successfully developed for molecular imaging and targeted therapy. The focus of the current chapter is to highlight the utilization of aptamers as targeting molecules for theranostic biomaterials. Aptamers are increasingly proving to be a powerful tool in research and drug development for various conditions, with minimal effect on healthy tissues by assuring more selective drug targeting and delivery and, thus, better therapeutic efficacy and potency.

APTAMERS

The word Aptamer is derived from the Latin “aptus”, which means “fit” and the suffix “- meros” which means “portion”. An Aptamer is a single-stranded DNA or RNA molecule [4] obtained through Systematic Evolution of Ligands by EXponential enrichment (SELEX). An Aptamer’s binding to its target is quite specific. Aptamers are also known as chemical antibodies, like antibodies; they

bind to targets with high affinity, modulate target function, deliver cargo to specific sites, and are used in diagnostic and analytical assays, whether in solution or immobilized forms. However, aptamers surpass their proteinaceous counterparts by their low immunogenicity, physical stability, facile chemical modification, and the relative ease of their large-scale synthesis at affordable costs with little or no batch-to-batch variation. These alluring properties have propelled aptamers to the forefront of therapeutic and diagnostic agent development as well as bio-sensing platform construction [3]. Numerous aptamers have been developed during the past 2 to 3 decades for a variety of medical applications, such as chemical sensors, imaging molecules, diagnostic assays, and active therapeutic molecules [4].

Aptamers can be generated using either DNA or RNA oligonucleotides. Both types act similarly as binding molecules. However, RNA aptamers are widely believed to produce more complex 3D structures, whereas DNA aptamers are more stable and have a lower cost of production [8]. Although several unmodified DNA and RNA aptamers have been successfully selected for different targets, these aptamers are highly susceptible to degradation by nucleases and rapid clearance from the circulation. Therefore, several chemical modifications have been applied and investigated at different parts of the aptamers sequences. These chemical modifications were shown to improve the physio-chemical properties of the aptamers, increasing their stability, bioavailability, diversity, and establishing hydrophobic interactions [3, 9].

The backbone (phosphate/ribose) of the aptamer sequence is a common site for chemical modification. A common modification is the introduction of the phosphorothiolated linkage instead of the normal phosphodiester linkage. However, thiolated aptamers may induce toxicity and nonspecific interactions. When it comes to RNA aptamers, ribose sugar is the preferred site for modifications. The ribonuclease A (RNase A) targets 2′-hydroxyl group of ribopyrimidines and breaks the phosphodiester bond in the sugar-phosphate backbone of RNA strands. Thus, substituting the 2′-hydroxyl group of ribopyrimidines by 2′-amino, 2′-fluoro, or 2′-O-methoxy groups lead to a significant increase in the stability of RNA. Several reports showed that the 2′-amino modification increases the RNA half-life in serum to 170 hours compared to 10 seconds of the unmodified RNA aptamers. However, the 2′- amino modification affect thermodynamic stability of the aptamer, thus hindering binding affinity [10]. On the other hand, 2′-fluoro modifications also increase stability with half-life around 90 hours in serum. This type of modification was used in Macugen® development [11]. Moreover, 2′-fluoro modifications showed better base pair stability and binding affinity of the aptamers [11]. Another interesting approach to develop nuclease-resistant and stable aptamers is through

modification with locked nucleic acids (LNA) or using L-bases (mirrored nucleobases) called Spieglemers, which can be selected using mirror-SELEX [12].

Although the small size of aptamers provides higher penetration ability, it also makes aptamers susceptible to high renal clearance, thus decreasing the bioavailability of aptamers. This problem was solved by conjugating aptamers with a variety of relatively high atomic mass molecules such as polyethylene glycol, proteins, organic and inorganic nanoparticles, sugars, and cholesterol [13]. Recently, introducing a protein-like side chain at the 5′-position of pyrimidines and the 8′-position of purines away from the hydrogen bonding face of the bases of aptamers gave them hydrophobic characteristics and increased the ability to bind with more epitopes. These ligands have very low dissociation rate constants and are named SOMAmers (Slow Off-rate Modified Aptamers) [14].

SELEX

Aptamers are selected with high target affinity and specificity using a screening technology termed Systematic Evolution of Ligands by EXponential enrichment (SELEX). This method generates DNA as well as RNA aptamers against the target of choice. The method is quite similar for both DNA and RNA, with the main difference being that RNA aptamers require additional steps of *in vitro* transcription and reverse transcription during SELEX. Aptamers versatility enables the utilization of aptamers against an ample variety of targets ranging from ions to whole living cells. A library of random dsDNA sequences is chemically synthesized to produce ssDNA library or undergo *in vitro* transcription to produce ssRNA library. The resulting sequences then compete through SELEX cycles. Each sequence in the synthesized library folds into a unique 3-D structure. Selection conditions such as ionic strength, pH, and temperature are set according to the intended application. A library of 10^{13} to 10^{15} different folded oligonucleotides sequences is incubated with the target of choice. Each sequence consists of constant domains at 5′- and -3′ ends, intended for primer annealing and amplification, and a random sequence in the middle. After incubation, unbound sequences are washed through and the bound sequences are eluted, amplified, and incubated with their target in iterative selection cycles. Selection parameters can be altered to yield the ideal aptamer affinity. Selection cycles are repeated until sequences with high affinity and specificity are isolated [15].

Several modifications of SELEX technology have emerged since aptamers were first discovered [7]. Cell-SELEX is one variation where aptamers are developed for targets existing on living cells. In cell-SELEX, bound sequences are introduced to extra steps of counter-selection where the library is incubated with

control cells related to target cells. Sequences that bind control cells in the counter selection are excluded. This increases aptamer specificity.

Another SELEX variation is *in vivo* SELEX. Aptamers are selected for targets that exist in living organisms instead of using isolated pure targets or individual cells. This technique overcomes some of the obstacles of aptamer selection *in vitro*. *In vivo* SELEX is similar in principle to conventional *in vitro* methods. Here, the library of sequences is injected into the peripheral vasculature of the living organism. Specific aptamers homed into targeted tissue are tracked and isolated. Indeed, *in vivo* SELEX has shown promising results for tissue selection which can be applied in different diseases, including cancers and virally infected cells [16 - 18].

APPLICATIONS OF APTAMERS

Diagnosis

Aptamers can be selected against a wide range of targets; therefore, they have been used as a tool for developing various diagnostic systems such as targeting ligands for imaging nanocarriers, optical aptasensors, and electrochemical aptasensors [19, 20]. Aptamers-guided imaging nanoparticles have been developed for screening and detection of cancer cells and pathogenic infections. This approach enables specific detection of targets in complex fluids (*e.g.* blood) and protects aptamers from being digested *via* nucleases. For imaging purposes, aptamers were successfully immobilized on the surfaces of different nanoparticles such as gold nanoparticles (AuNPs), magnetic NPs (MNP), graphenes, silica NPs (SiNP), and quantum dots (QDs) [12, 21 - 23]. For example, the Mucin 1 (MUC1) aptamers were conjugated to dye-doped silica nanoparticles and showed high selectivity and steady fluorescence signal toward MUC1(+) cell lines [24]. Developing biosensors is another interesting diagnostic approach for aptamers. Biosensors involving aptamers are called aptasensors and can be divided into optical and electrochemical aptasensores. For optical aptasensors, the aptamers can be labeled with reporters having fluorescence, luminophores, and enzymes or can be used label-free in optical resonance based detection methods [25, 26]. For example, an anti- adenosine DNA aptamer has been modified with fluorescein dye and hybridized with a 12-mer complementary DNA at the 5′- end and conjugated to a quencher dye at the 3′-end. This aptamer was immobilized onto magnetic microparticles to sense adenosine in serum at micromolar level. The binding with adenosine stimulates the release of the quencher strand and recovers the fluorescence signal [27].

The other type of aptasensors is electrochemical aptasensors. Typically, these aptasensors monitor analyte binding events through electrochemical current variation produced from the interaction between the aptasensor and the analyte in which the electrode surface is used as a dais to immobilize the sensing aptamer. For instance, a short anti-thrombin DNA sequence labeled with methylene blue (MB) was immobilized on a gold surface for detection of thrombin in serum. The principle is based on the conformational changes resulting from thrombin binding to the immobilized aptamer. These conformational changes induce alterations in the current that can be detected by electrochemistry-based methods [28, 29].

Therapy

Several aptamers have been developed to act as drugs. These therapeutic aptamers bind to their targets and modulate their biological activity. They proved effective against many diseases like cancer, cardiovascular disease, and infectious diseases. There are several routes of administration of therapeutic aptamers, including intravenous (IV), subcutaneous (SC), and intravitreal (IVT) administration [30]. The first therapeutic aptamer approved by US FDA in December 2004 was Macugen® (Pegaptanib sodium). Pegaptanib is an RNA aptamer that binds and blocks the function of vascular endothelial growth factor (anti-VEGF) and has been used for the treatment of age-related macular degeneration (AMD). The 28 nucleotides that made Pegaptanib are primarily synthesized from 2′-fluoro nucleotides and modified with 2′-O-methyl. To further increase the stability of Pegaptanib, it has been synthesized with 2′-deoxy-2′-fluoro cytidine and uridine and equipped with inverted deoxythymidine to cap the 3′-end. Moreover, to decrease renal clearance and increase half-life, Pegaptanib has been PEGylated by conjugating polyethylene glycol (PEG) to the 5′ end of the aptamer [11, 31]. On the same side, the anti-nucleolin aptamer (AS1411) is one of the most characterized therapeutic anticancer aptamers. AS1411 is a quadruplex-forming guanine-rich (G-rich) DNA aptamer composed of 26-nucleotides that bind with nucleolin expressed on cancer cells and inhibit cell proliferation through destabilizing the activity of important signaling proteins, including BCL-2 and NF-κb [32, 33]. Aptamers therapeutic potency has also been proved as anti-infective agents for many bacterial and viral infectious agents such as Mycobacterium tuberculosis [34], *Salmonella* [35], *Listeria monocytogenes* [36], HIV-1 [37], HCV [38], and *Influenza* [39]. For instance, many potential therapeutic aptamers have been developed against HIV by targeting proteins, receptors, and enzymes involved in HIV infection. For example, anti-gp120 aptamers that target viral surface protein (gp120) displayed the ability of viral inhibition by binding and neutralizing gp120 [37]. In addition, aptamers have been widely used in therapy of cardiovascular diseases; for example, aptamers

were used as anti-thrombotic agents, such as REG1 anti-thrombotic aptamer [40] which binds to and blocks Factor IXa [41].

APTAMERS TARGETING FOR THERANOSTICS BIONANOMATERIALS

Theranostics bionanomaterials involve the development of delivering nanomaterials that combine diagnostic and therapeutic functions within a single nanosystem. Thus, a nanosystem that can co-deliver a therapy and an imaging agent is extremely important for molecular medicine, in which molecular imaging with simultaneous diagnosis and therapy will provide the multimodality needed for accurate diagnosis and targeted therapy [42].

A theranostic nano-platform with a targeted and efficient drug transport system would improve specificity and enhance therapeutic efficacy. In addition, aptamers, as specific recognition elements, have been utilized for active targeted transport of many conventional chemotherapeutic drugs [43]. Recent biotechnological expansions have led to a variety of targeted drug transport (TDT) strategies based on aptamer–drug conjugates or aptamer–nanomaterial assemblies [44, 45].

Among all targeted nanocarriers, smart nanostructures combining tumor targeting, tumor therapy, and tumor imaging are the most beneficial and provide more specific therapeutic protocols, have a better prognosis, and simultaneous improvement of treatment efficiency and reduction in side effects [46].

Targeted Delivery and Imaging of Anthracycline Nanomaterials

Anthracyclines are a group of hydrophilic drugs that interact with DNA by intercalation, inhibiting replication, and deregulating gene expression [46]. Most aptamer-targeted theranostic studies are concerned with the co-delivery of imaging agents and anthracycline drugs, mainly doxorubicin (DOX). These drugs were firstly loaded into certain aptamer conjugated-nanoparticles labeled for detection by imaging agents.

Zhu *et al*. developed sgc8 aptamer-based DNA nanotrains (aptNTrs) by hybridization chain reaction (HCR) and utilized them to carry doxorubicin for cancer therapy purposes. Moreover, fluorophores on nanotrains and DOX fluorescence upon release allowed intracellular signaling of nanotrains and drugs. Such results make aptNTrs a promising targeted drug transport platform for cancer theranostics [43].

Targeted chemotherapy and magnetic resonance imaging of cancer cells *in vitro* has been achieved using a smart multifunctional pH-sensitive nanostructure (SMN) constructed from a porous hollow magnetite nanoparticle (PHMNP), a heterobifunctional PEG ligand, and an sgc8 aptamer (Fig. **1**). The PHMNPs were prepared through a three-step reaction. First, the 13 nm iron-magnetite core shell nanoparticles (IMNPs) were produced by thermal decomposition of iron pentacarbonyl ($Fe(CO)_5$). Second, 16 nm hollow magnetite nanoparticles (HMNPs) were produced through the oxidation IMNPs in the presence of N-oxide (Me_3NO). Third, the PHMNPs were produced by acid etching at high temperatures in the presence of oleic acid. Finally, the PHMNPs were loaded with Dox while being PEGylated and functionalized with sgc8 aptamers to produce smart multifunctional nanostructure (SMN) [47].

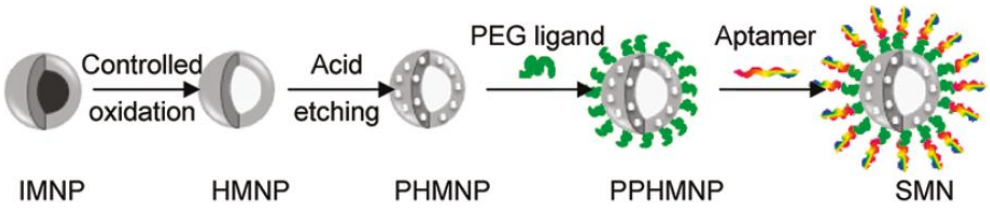

Fig. (1). Schematic illustrating the synthesis of SMNs. Reprinted with permission from ref [47]. Copyright (2011) American Chemical Society.

A multifunctional, thermally cross-linked superparamagnetic iron oxide nanoparticles (TCL-SPION) functionalized with PSMA aptamer was developed for detecting prostate cancer (PCa) cells, and deliver targeted chemotherapeutic agents, DOX, directly to the PCa cells. The formulated SPION–Apt bioconjugates have been utilized to combine magnetic resonance-based molecular imaging and selective chemotherapy in PCa cells [48].

Three years later, Kyung *et al*. also synthesized and investigated the thermally cross-linked superparamagnetic iron oxide nanoparticles (TCLSPIONs) conjugated to the CG-rich duplex PSMA aptamer loaded with DOX as prostate cancer-specific nano-theranostic agents. These agents were able to detect a prostate tumor *in vivo* by magnetic resonance imaging (MRI) and selective delivery of drugs to the tumor tissue simultaneously. The prepared nano-theranostic agents exhibited superior binding towards prostate-cancer cells (LNCaP, PSMA +) in both *in vitro* and *in vivo* when analyzed by T_2 -weighted MRI on the LNCaP xenograft mouse model [49]. Furthermore, Mosafer *et al*. co-encapsulated SPIONs and DOX into the poly (lactic-coglycolic) acid (PLGA)-based nanoparticles which then were targeted with AS1411 aptamer (Apt) against murine C26 colon carcinoma cells for theranostic purposes. The Apt conjugation

to NPs (Apt-NPs) enhanced cellular uptake of Dox in C26 colon carcinoma xenograft model. Moreover, Apt-NPs enhanced the contrast of MRI in tumor sites [50].

On the other side, Bagalkot *et al* reported a multifunctional nanosystem composed of quantum dot (QD)- A10 RNA aptamer (Apt)-Dox conjugate [QD-Apt(Dox)] as a targeted cancer imaging, therapy, and sensing system (Fig. **2**). The targeted QD imaging system (QD-Apt) was capable of detecting and imaging prostate cancer cells. This multifunctional nanoparticle system can deliver Dox to the targeted prostate cancer cells and sense the delivery of Dox by activating the fluorescence of QD, which concurrently images the cancer cells. The release of Dox from the QD-Apt (Dox) conjugate induces the recovery of fluorescence from both QD and Dox ("ON" state), thereby sensing the intracellular delivery of Dox, and enabling the synchronous fluorescent localization and killing of cancer cells [51].

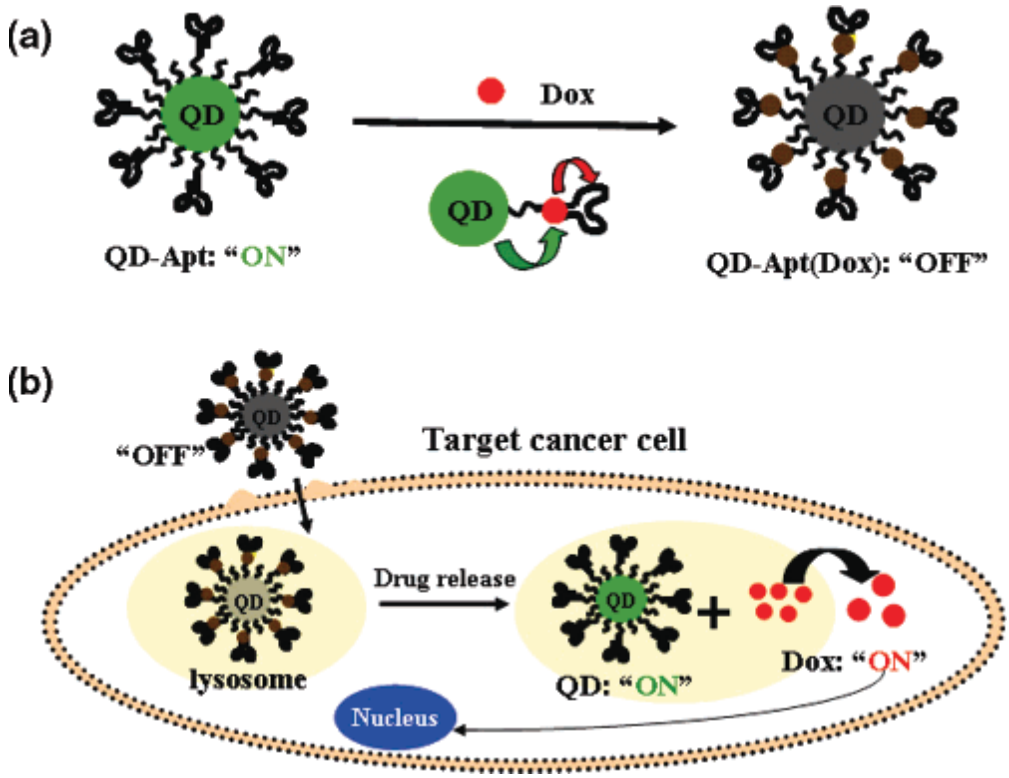

Fig. (2). (a) Schematic illustration of QD-Apt (Dox) Bi-FRET system. **(b)** Schematic illustration of specific uptake of QD-Apt (Dox) conjugates into target cancer cell through PSMA mediates endocytosis. Reprinted with permission from ref [51]. Copyright (2007) American Chemical Society.

Savla *et al*. reported the design and delivery of a tumor-targeted, pH-responsive quantum dot-Mucin 1 aptamer-doxorubicin (QD-MUC1-DOX) conjugate for imaging and treatment of ovarian cancer cells. These conjugates were shown to be stable in the general circulation at a neutral and slightly basic pH but undergo rapid hydrolysis in the acidic environment of endosomes. However, the developed QD-MUC1-DOX conjugate showed higher cytotoxicity than free DOX in multidrug-resistant cancer cells and preferentially accumulated in ovarian tumors. In addition, quantum dots also have the potential as carriers of drugs and as *in vivo* imaging agents [52].

Additionally, a novel theranostic nanoprobe was designed from polyvalent carboxymethyl cellulose modified magnetic nanoparticles (CMC-MNPs), which can be functionalized with cancer-specific molecule (EpCAM aptamer) and loaded with an anticancer drug (DOX). CMC-MNPs nanoprobe showed efficient *in vitro* MR imaging and targeted drug delivery with comparable treatment efficacy toward cancer cells [53].

Interestingly, a smart split aptamer-based activatable theranostic probe (SATP) was designed for cancer-activated *in vivo* imaging and *in situ* drug release (Fig. **3**). The SATP assembly was made of two split parts of the Sgc8c aptamer (Apt-L) that are linked *via* a short DNA (S) linker labeled with a fluorophore (F) and hybridized with a complementary short DNA strand. Dox was used as a model drug-loaded between the GC-rich double-helix regions of the linker/short DNA strand. In the free-state, the SATP is double-stranded with signal inactivated and DOX loaded. Once encountering the target cell, the SATP can disassemble based on aptamer binding-triggered conformational alteration, thus leading to fluorescence activation and DOX release. The SATP realized highly-specific drug delivery and CEM cancer cell killing as a result of target-triggered *in situ* release. Both therapeutic and imaging functions were validated *in vitro*. Moreover, *in vivo* and *ex vivo* investigations also revealed that the SATP was specifically activated by CEM tumors inside mice, and contrast-enhanced imaging was achieved in 5 minutes, indicating rapid diagnosis and timely therapeutic effect of SATP [54].

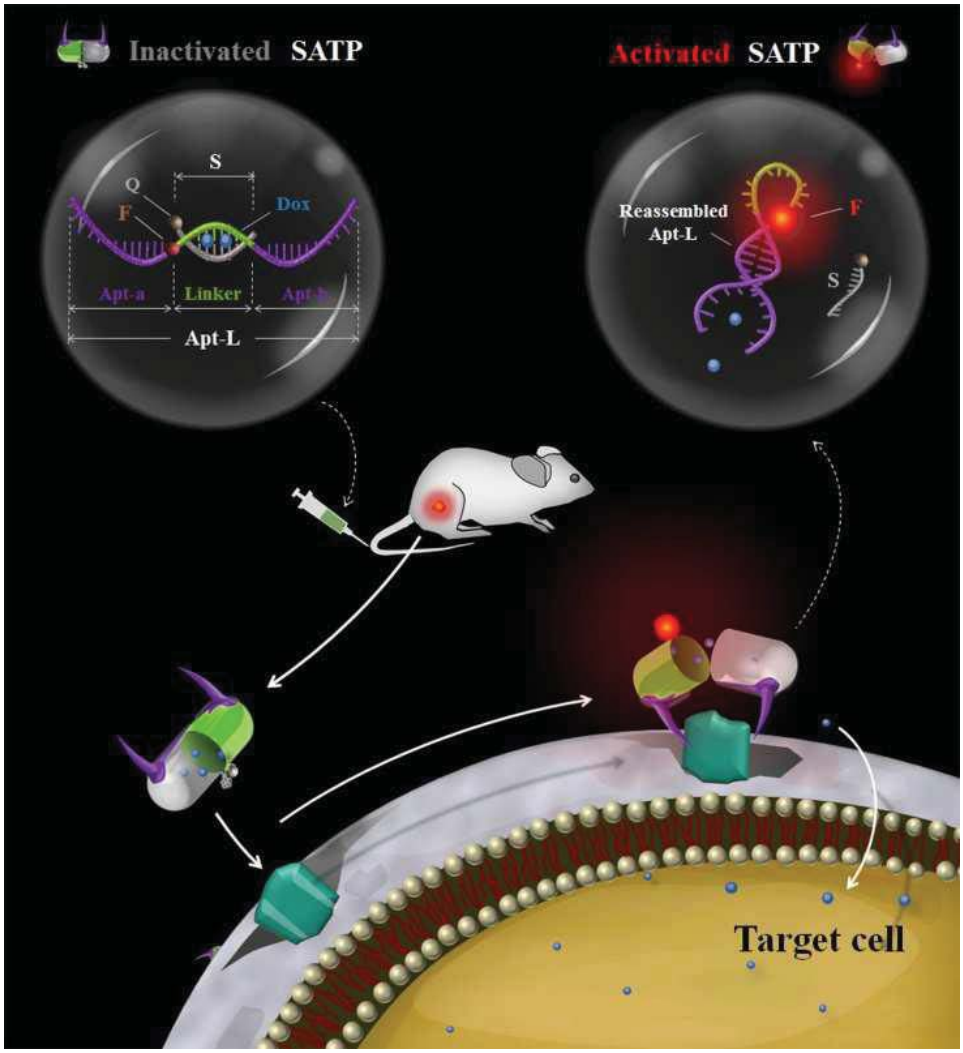

Fig. (3). The structure and working principle of split aptamer-based activatable theranostic probe (SATP) in cancer theranostics. Reprinted with permission from ref [54]. Copyright (2016) American Chemical Society.

Moreover, anti-MUC1 aptamer-capped mesoporous silica nanoparticles loaded with safranin O and DOX and radiolabeled by ^{99}Tc, detected by single-photon emission computed tomography (SPECT), were synthesized for targeted delivery, bio-imaging and treatment of MDA-MB-231 breast cancer cells that overexpress glycoprotein MUC1. This theranostic nanoparticle showed a remarkable tumor signaling and accumulation with a marked reduction in cells viability related to the release of the entrapped doxorubicin [55].

On the other hand, Kim *et al*. established a theranostic nanosystem for targeting prostate cancer. They functionalized the surface of gold nanoparticles (GNPs) with a prostate-specific membrane antigen (PSMA) RNA aptamer loaded with DOX (Fig. **4**). The targeted computed tomography (CT) imaging system was capable of specific imaging of LNCaP prostate cancer cells that express the PSMA protein. The resulting nanosystem showed more than 4-fold greater CT intensity with enhanced doxorubicin potency for LNCaP cells compared to PSMA-negative PC3 cells [56].

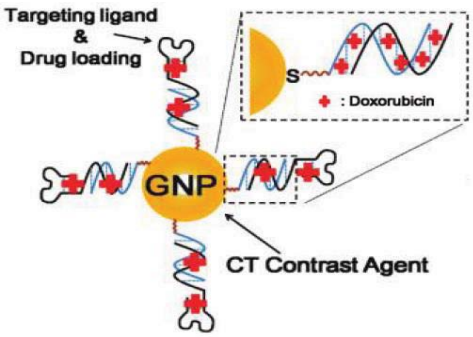

Fig. (4). DOX-loaded PSMA aptamer-conjugated GNPs. Reprinted with permission from ref [56]. Copyright (2010) American Chemical Society.

Also, a smart nanoparticle was established by conjugating gold nanocluster (AuNC) with cyclic Arg-Gly-Asp (cRGD) peptide and aptamer AS1411 (Apt) (AuNC-cRGD-Apt) for dual targeting of tumor cells. The AuNC-cRGD-Apt was further functionalized with near-infrared (NIR) fluorescent dye (MPA) and loaded with DOX for cancer diagnosis and therapy. AuNC-MPA-cRGD-Apt displayed low cytotoxicity and favorable tumor-targeting capability at both *in vitro* and *in vivo* levels with high potential for tumor imaging [57].

Lin *et al*. also utilized the excellent imaging properties of QDs in designing a theranostic nanosystem. They developed daunorubicin (DNR)-loaded MUC1 aptamer-near infrared (NIR) CuInS2 quantum dot (DNR–MUC1–QDs) conjugates, which were used as imaging and sensing systems to target prostate cancer cells *in vitro* [58].

The superparamagnetic iron oxide nanoparticles SPION have also been utilized for delivery of epirubicin *in vivo* by using the anti-MUC1 aptamer combined with epirubicin and SPION nanoparticles. Epirubicin holds fluorescence property that could be quenched by interaction with the aptamer. This nanocomplex system significantly inhibited the *in vivo* tumor growth of colon cancer cell xenograft compared with the treatment with non-targeted epirubicin [59].

Targeted Delivery and Imaging of Taxane Nanomaterials

Taxane drugs, such as paclitaxel and docetaxel, are hydrophobic chemotherapy agents. These drugs disrupt the microtubule functions that are essential for cell division [46]. Some aptamer-targeted theranostic-nanoparticles have been generated for the co-delivery of taxanes with imaging agents. For example, an aptamer AS1411-functionalized poly (l-γ-glutamyl-glutamine)-paclitaxel (PGG-PTX) nanoconjugates loaded with paclitaxel and fluorescence dyes, DiO and DiR (AS1411-PGG-PTX) were established by Luo *et al.* for targeted delivery against human U87 MG glioblastoma cells. *In vivo* fluorescence imaging and tissue penetration demonstrated a higher distribution of AS1411-PGG-PTX in tumor tissues as compared with PGG-PTX. The AS1411-PGG-PTX nanoconjugates presented the best anti-glioblastoma effect *in vivo* with prolonged median survival and high tumor cells apoptosis as compared with other groups [60].

Gao *et al*. utilized a phage-displayed TGN peptide and AS1411 aptamer to functionalize the surface of poly(ethylene glycol)-poly(ε-caprolactone) (PEG-PCL) nanoparticles (AsTNPs) which are loaded with docetaxel and fluorescence dye to target orthotopic brain tumor model. Fluorescence imaging demonstrated that the AsTNPs provided the highest tumor distribution and tumor/normal brain ratio and penetrate the endothelial monolayers and tumor cells to reach the core of the tumor spheroids. The docetaxel-loaded AsTNPs present a valuable tool for glioma imaging and therapy [61]. Gao and colleagues again utilized the same docetaxel-loaded nanoparticles that only functionalized with a selected GMT8 DNA aptamer and a fluorescent dye for glioblastoma therapy. *In vitro* and *in vivo* fluorescence imaging and biodistribution studies demonstrated that these functionalized nanoparticles enhanced intracellular drug delivery and tumor spheroid penetration, which could be used in the future to target glioblastoma and accumulate at the tumor site [62].

Also, Tao *et al*. designed an anti-nucleolin aptamer conjugated-nanoparticles of cholic acid-functionalized with copolymer consisting of PLGA and vitamin E loaded with docetaxel and a near-infrared heptamethine fluorescent dye (Apt-p--DTX/NPs). Both *in vitro* and *in vivo* imaging and biodistribution studies verified that the Apt-pD-DTX/NPs could achieve significantly high targeting efficiency and enhanced therapeutic effects compared with clinical Taxotere® or NPs without functional modification, and represent the Apt-pD-DTX/NPs as a potentially qualified drug delivery system to target breast cancer [63].

Targeted Delivery and Imaging of Nucleic Acid Drugs Nanomaterials

A liposomal formula of aptamer-conjugated liposomes containing Q-dots and siRNA molecules (Apt-QLs) was optimized. The anti-EGFR Apt-QLs exhibited

remarkable EGFR-dependent therapeutic anticancer activity in addition to siRNAs delivery as well as fluorescence imaging, which have been analyzed in cultured cancer cells and tumor xenografts in mice. EGFR-targeting QLs showed enhanced delivery to target cancer cells, resulting in more effective gene silencing and enhanced tumor imaging compared to non-targeting control QLs. The results implied that the formulation of Apt-QLs could be widely utilized as a carrier for tumor-directed gene delivery and bio-imaging agents [64, 65].

Kim *et al*. developed a cancer-targeting theranostic probe using an AS1411 aptamer and miRNA-221 molecular beacon (miR-221 MB)-conjugated magnetic fluorescence (MF) nanoparticle (MFAS miR-221 MB) to simultaneously target cancer tissue, image intracellularly expressed miRNA-221, and treat miRNA-22--involved carcinogenesis. AS1411 aptamer-conjugated MF (MFAS) nanoparticles displayed high selectivity and delivery into various cancer cell lines. The miR-221 MB detached from the MFAS miR-221 MB in the cytoplasm of C6 cells clearly imaged miRNA-221 biogenesis and simultaneously resulted in antitumor therapeutic effects by inhibiting miRNA function, indicating a successful astrocytoma-targeting theranostics [66].

Targeted Delivery, Imaging, and Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is an emerging type of therapy that uses light-activatable molecule, a photosensitizing chemical substance, to produce highly cytotoxic reactive oxygen species when excited by light that causes death of target cells. The procedure involves the administration of a photosensitizing substance followed by irradiation at a wavelength corresponding to an absorbance band of the photosensitizer. For clinical purposes, the photosensitizing agent is typically administered intravenously, intraperitoneally, or topically, followed by an exposure to light delivery system suitable for the anatomical site being treated [67, 68]. PDT consists of three main elements: a photosensitizer, light and oxygen, where together they initiate a photochemical reaction that ends in the generation of a highly reactive oxygen species such as superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) that provoke significant toxicity leading to cell death *via* apoptosis or necrosis [69, 70]

PDT is considered a minimally invasive approach characterized by high effectiveness in treating cancer cells and a lack of intrinsic or acquired resistance mechanisms [69]. However, PDT cannot discriminate the diseased cells from the normal cells. Integrating PDT and imaging nanoparticle is an approach to overcome this problem [71] that proved to be a valuable tool for understanding PDT tissue interactions, treatment monitoring, diagnosis, and dosimetry [72]. A

more specific and selective therapy was introduced with aptamer conjugated-nanoparticle PDT combined with imaging agents (Fig. **5**).

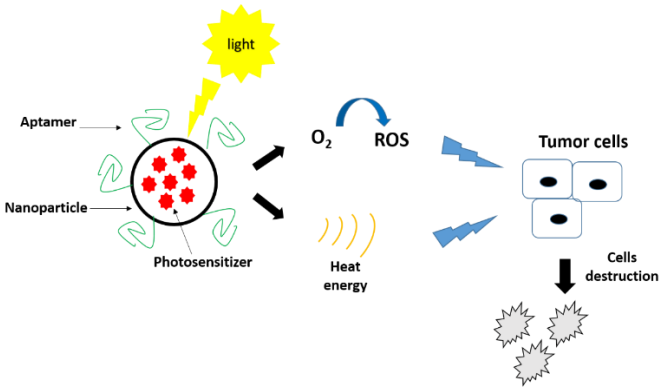

Fig. (5). Aptamer functionalized nanoparticles for photodynamic or photothermal therapy of tumor cells.

For example, Mallikaratchy *et al*. designed a photosensitizer known as chlorin e6 (Ce6) which was chemically conjugated to a selected TD05 aptamer that only recognizes Ramos cells, a Burkitt's lymphoma cell line. The aptamer-photosensitizer conjugates (APS) followed by irradiation of light enhanced the selectivity of photodynamic therapy toward cancer cells *in vitro* [73]. A similar approach was recently used to target cancer cells overexpressing nucleolin receptor using anti-nucleolin aptamer-chlorin Ce6 conjugate and was shown to improve tumor treatment using endo-laparoscopic PDT [74]. Again, anti-nucleolin aptamer was used to deliver porphyrin photosensitizer encapsulated in virus nanoparticles into MCF-7 breast cancer cells *in vitro*, and showed higher phototoxicity compared with MCF cells treated with virus particles not conjugated with targeted aptamers [75]. Furthermore, nanomicelles functionalized by aptamers, selected specifically against MDA-MB-231 human breast cancer cells, and encapsulating a NIR photosensitizer and a pH-activatable fluorescent probe, which lights up when reaching lysosomes, were used *in vitro* to target breast cancer cells. It has been demonstrated that upon NIR irradiation, NIR photosensitizer-mediated generation of reactive oxygen species provoked lysosomal destruction and subsequent cancer cell death [76].

Targeted delivery, imaging, and Photothermal therapy (PTT)

Photothermal therapy (PTT) is an extension of photodynamic therapy in which a photosensitizer is excited with light radiation to release energy (heat) that can be used for the treatment of various medical conditions and killing of tumor cells [77]. In PTT, usually, NIR irradiation is used to illuminate the target cancer cells either topically or interstitially through an optical fiber, and then the light energy

is converted into heat energy (temperature ≥ 41°C) to release chemotherapeutic drugs and/or induce cellular necrosis or apoptosis [78]. Over time, this process leads to either partial or complete ablation of the target tissue. PTT exhibits unique advantages in cancer therapy, including minimal invasiveness and precise spatial-temporal selectivity. Also, the external light stimulus used in PTT, can be easily regulated, focused, and remotely controlled, which enables better-targeted therapy that leads to less damage to surrounding healthy tissues [79].

Aptamers conjugated nanoparticles system, which co-deliver a therapy and an imaging agent, was used to improve light delivery and guide PTT to specifically target tumor tissues and decrease unwanted effects on normal cells. Li *et al*. reported the use of Sgc8 aptamer-conjugated Au-coated Fe_3O_4 nanoroses for selective theranostic effect against CCRF-CEM cancer cells. Fe_3O_4 nanoroses served as a magnetic resonance imaging (MRI) agent, while the photothermal effect is achieved through near-infrared absorption by the gold shell, which facilitated the release of the chemotherapeutic anticancer drug DOX [80].

Kolovskaya *et al*. demonstrated the production of anticancer DNA aptamer-conjugated to spherical gold nanoparticles, which showed high efficacy in eradicating Ehrlich carcinoma in tumor-bearing mice after photothermal treatment [81]. In another study, NIR light-absorbing hollow gold nanocages were synthesized and conjugated with aptamer that specifically binds nucleolin to achieve cancer-targeted photothermal therapy. The generated anti-nucleolin aptamer-gold nanocage exhibited high binding and cellular uptake in cancer cells. Overall, the synthetic antitumor aptamers direct the nanoparticles precisely to the target cells and selectively eliminate cancer cells after the subsequent light excitation of the sensitizer agent [82]. Also, a fluorescent-labeled aptamer, Cy5-labeled aptamer Sgc8c (Cy5-Sgc8c), conjugated on the surface of Cu-Au alloy nanostructures were generated to achieve active PTT targeting and signal output simultaneously. Remarkably, the generated nanosystem exhibited successful selective fluorescence imaging and NIR photothermal therapy, in which robust target recognition and stable signal output for molecular imaging were observed. Also, the system was efficient in killing cancer cells both in cancer cell lines *in vitro* and mice models *in vivo* [83].

On the same side, Fan *et al*. demonstrated a multifunctional nanosystem based on S6 aptamer-conjugated magnetic core-plasmonic shell nanoparticles for diagnosis, isolation, and photothermal destruction of K-BR-3 human cancer cells (Fig. **6**). They found that bioconjugated magnetic/plasmonic nanoparticles can be used for imaging and magnetic separation of SK-BR-3, HaCaT, or LNCaP prostate cancer cell lines. A targeted photothermal experiment using 670 nm light at 2.5 W/cm2 for 10 minutes resulted in selective irreparable cellular damage to most of the

cancer cells [84]. Fan's group also developed a theranostic plasmonic shell−magnetic core star shape nanomaterial-based approach for the targeted isolation of rare tumor cells from the whole blood sample, followed by diagnosis and photothermal destruction. They showed that Cy3 tagged S6 aptamer-conjugated theranostic plasmonic/magnetic nanoparticles can be used for fluorescence imaging and magnetic separation of SK-BR-3 cells, which overexpress HER2 proteins. They also demonstrated that irradiation of cancerous cells bound to S6 aptamer conjugated magnetic/plasmonic nanoparticles by NIR light selectively kills most of the cancer cells within 10 min [85].

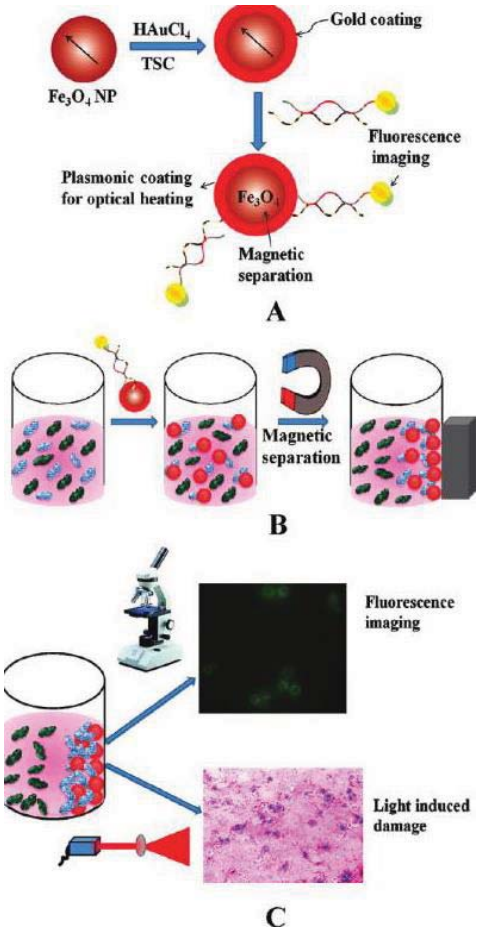

Fig. (6). Schematic representation showing **(A)** The synthesis of S6 aptamer-conjugated multifunctional magnetic core-gold shell nanoparticles, **(B)** The separation of specific cancer cells using S6 aptamer-conjugated plasmonic/magnetic nanoparticles, **(C)** The selective fluorescence imaging and targeted photothermal destruction of specific cancer cells. Reprinted with permission from ref [84]. Copyright (2012) American Chemical Society.

In a slightly different approach, Shi *et al*. developed a theranostic model based on a switchable aptamer and a fluorescence-labeled complementary DNA sequence as imaging elements. In this model, a switchable S6 aptamer specific for A549 lung cancer cells was first hybridized with a fluorescence-labeled complementary DNA sequence and then grafted onto the surface of Au@Ag/Au NPs, which were used as photothermal agents and fluorescence quenchers. In the absence of A549 target cells, fluorescence was quenched by Au@Ag/Au NPs due to their close proximity to the DNA. However, upon the aptamer binding to its target cells, its conformation changed and fluorescence was activated. Evaluation of these conjugates in *in vivo* models of lung cancer revealed that, for mice treated with theranostics, a prominent tumor fluorescence 5 min after the injection was observed, and subsequent photothermal therapy resulted in a significant level of tumor necrosis [86].

Other Targeted Drug Delivery and Multimodal Imaging

Potential anticancer agents, rather than anthracyclines and taxanes, were also co-delivered with imaging agents into aptamer conjugated-theranostic nanoparticles. Nutlin-3a is a potent and selective small-molecule MDM2 antagonist that has shown considerable promise in pre-clinical studies leads to cell cycle arrest and apoptosis [87]. Bovine lactoferrin (bLf) is a naturally occurring protein that has attracted attention as a safe anticancer agent capable of inducing apoptosis [88]. Besides their targeting activity, aptamers are a class of potential therapeutic agents [7] whereby the majority of the clinically tested aptamers act as inhibitors, by binding and inhibiting enzyme activities or protein-protein interactions [89]. A multifunctional nanotheranostic system was formulated by conjugating EpCAM aptamers and imaging quantum dots (QD) onto the surface of PLGA NPs that were pre-loaded with a chemotherapeutic agent nutlin-3a. This nutlin-3a-loaded apt-QD-PLGA nanosystem demonstrated better accumulation and deeper penetration into QD-visualizing EpCAM+ breast and ovarian cancer spheroids than the untargeted NPs counterparts and showed promising therapeutic effects against EpCAM+ tumor cells. Moreover, this nanotheranostic system served as a superlative bio-imaging modality both in 2D monolayer culture and tumor spheroid model [90].

Additionally, a fluorescent silica nanoparticles (FSiNPs) theranostic system decorated by the Cy5.5 fluorophore tagged mouse endgolin (mEND) aptamer (YQ26) has been developed by Tan *et al*. for *in vivo* tumor imaging, treatment, and monitoring (Fig. **7**). Both *in vitro* and *in vivo* experiments clearly verified that YQ26-FSiNPs achieved high targeting efficiency and therapeutic effects *via* aptamer YQ26-mediated binding to endoglin (END) molecule. The YQ26-FSiNPs

theranostic system provided an effective strategy for clinical tumor-targeted imaging and treatment with high sensitivity and specificity for mEND *in vitro* and tumor vasculature *in vivo* [91]. In another study, a DY647 fluorophore was conjugated to the 5'-end of the EpCAM aptamer to facilitate molecular imaging. This conjugate possesses a number of superior attributes over monoclonal EpCAM antibodies in terms of tumor penetration, accumulation, and retention and is consistent with the criteria of *in vivo* imaging agents that make it a promising active targeting-based theranostics for both cancer therapy and *in vivo* imaging [92].

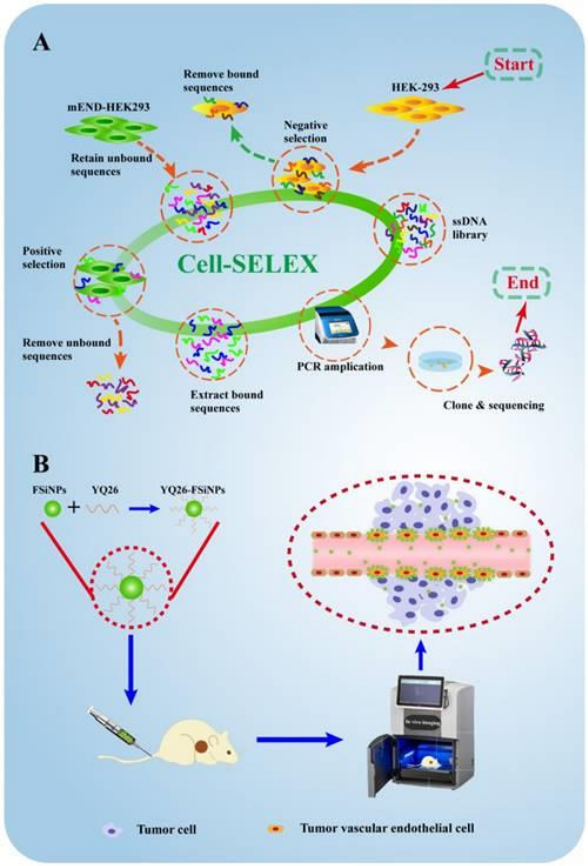

Fig. (7). Schematic illustration for selection and application of mouse endoglin aptamer. (A) The systematic evolution of DNA aptamers against mEND-HEK293 cells by Cell-SELEX. (B) The resulting aptamer YQ26 is conjugated to near-infrared fluorescent silica nanoparticles for imaging tumor vessels. Reprinted from ref [91]. Copyright (2017) Ivyspring International Publisher.

Recently, Roy *et al*. have investigated for the first time a novel combination of drug delivery system loaded with bovine lactoferrin (bLf) anticancer drug with a multimodal imaging efficacy of Fe_3O_4 nanoparticles (NPs). The nanoparticles

were synthesized from chitosan calcium phosphate and targeted with locked nucleic acid (LNA) aptamers against epithelial cell adhesion molecules (EpCAM) and nucleolin biomarkers. The nanoformulation showed a promising anticancer efficacy with complete regression of the tumors was observed for 70% of mice fed the nanoparticles and three-fold lower tumor recurrence after 30 days compared to those injected with non-targeted nanoparticles. Moreover, an exceptional tumor-targeting ability was observed by multimodal imaging systems using near-infrared (NIR) imaging, magnetic resonance imaging (MRI), and computerized tomographic (CT) techniques [93]. A detailed summary of aptamer-targeted theranostic nanomedicines described in this chapter is listed in Table **1**.

Table 1. A detailed summary of aptamer-targeted theranostic nanomedicines.

Target	Aptamer	Nanomaterial	Labeling	Imaging	Therapy	Reference
PTK7	sgc8	DNA nanotrains	Doxorubicin	Fluorescence	Doxorubicin	[6]
PTK7	sgc8	Porous hollow magnetite nanoparticle(PHMNP)	Iron-magnetite core	MRI	Doxorubicin	[7]
PSMA	A10	TCL-SPION	Iron oxide	MRI	Doxorubicin	[8]
PSMA	A10	TCL-SPION	Iron oxide	MRI	Doxorubicin	[9]
Nucleolin	AS1411	TCL-SPION and PLGA	Iron oxide	MRI	Doxorubicin	[10]
PSMA	A10	Quantum dot (QD)	QD	Fluorescence	Doxorubicin	[11]
Mucin	MUC1	Quantum dot (QD)	QD	Fluorescence	Doxorubicin	[12]
EpCAM	EpCAM	Polyvalent carboxymethyl cellulose modified magnetic nanoparticles (CMC-MNPs),	Iron oxide	MRI	Doxorubicin	[13]
PTK7	Sgc8c	Two split parts of Sgc8c aptamer with a complementary short DNA strand	Doxorubicin	Fluorescence	Doxorubicin	[14]
Mucin	MUC1	Mesoporous silica nanoparticles	^{99}Tc	Spect	Doxorubicin	[15]
PSMA	A10	Gold nanoparticles (GNPs)	Au	CT	Doxorubicin	[7]
Nucleolin	AS1411	Gold nanocluster (AuNC)	MPA dye	Fluorescence	Doxorubicin	[17]
Mucin	MUC1	Quantum dot (QD)	QD	Fluorescence	Daunorubicin	[18]
Mucin	MUC1	SPION	Iron oxide and Epirubicin	Fluorescence	Epirubicin	[19]

(Table 1) cont.....

Target	Aptamer	Nanomaterial	Labeling	Imaging	Therapy	Reference
Nucleolin	AS1411	Poly (l-γ-glutam-l-glutamine)	Fluorescence dyes, DiO and DiR	Fluorescence	Paclitaxel	[20]
Nucleolin	AS1411	PEG-PCL polymeric nanoparticle	Fluorescence dyes, DiO and DiR	Fluorescence	Docetaxel	[21]
U87 cells	GMT8	PEG-PCL polymeric nanoparticle	Fluorescence dyes, DiO and DiR	Fluorescence	Docetaxel	[22]
Nucleolin	AS1411	Cholic acid functionalized with copolymer consisting of PLGA and vitamin E	Near infrared heptamethine fluorescent dye	Fluorescence	Docetaxel	[23]
EGFR	EGFR	Liposomes containing Q-dots	QD	Fluorescence	siRNA	[24, 25]
Nucleolin	AS1411	Magnetic (cobalt ferrite core coated with a silica shell)	Rhodamine and Cy 5.0	Fluorescence	miRNA-221	[26]
Ramos cells	TD05	Molecular construct of a photosensitizer and an aptamer	Chlorin e6	Fluorescence	PDT	[33]
Nucleolin	AS1411	Molecular construct of a photosensitizer and an aptamer	Chlorin Ce6	Fluorescence	PDT	[34]
Nucleolin	AS1411	Virus nanoparticles	Porphoryn	Fluorescence	PDT	[35]
MDA-MB-231 cells	Apt S1	Nanomicelles	NIR photosensitizer, R16FP	Fluorescence	PDT	[36]
PTK7	Sgc8	Au-coated Fe3O4 nanoroses	Fe3O4	MRI	PTT and DOX	[40]
Nucleolin	AS1411	Spherical gold nanoparticles	Gold nanocages	Fluorescence	PTT	[41]
PTK7	Sgc8c	Cu-Au alloy nanostructures	Cy5	Fluorescence	PTT	[43]
SK-BR-3 cell	S6 aptamer	Magnetic core-plasmonic shell nanoparticles	Cy3	Fluorescence	PTT	[44]
SK-BR-3 cell	S6 aptamer	Plasmonic shell−magnetic core star shape nanomaterial	Cy3	Fluorescence	PTT	[45]
A549 and HER2 cells	S6 aptamer	Au @ Ag/Au NPs	Ag/Au and Cy5	Fluorescence	PTT	[46]
EpCAM	EpCAM	Quantum dots (QD) onto PLGA NPs	QD	Fluorescence	nutlin-3a	[51]

(Table 1) cont.....

Target	Aptamer	Nanomaterial	Labeling	Imaging	Therapy	Reference
mouse endgolin (mEND)	YQ26	Silica nanoparticles	Cy5.5	Fluorescence	YQ26 aptamer	[52]
EpCAM	EpCAM	EpCAM-DY647 fluorophore conjugate	DY647 fluorophore	Fluorescence	EpCAM	[53]
EpCAM and Nucleolin	Locked nucleic acid (LNA)	Fe3O4 nanoparticles	Fe3O4	NIR, MRI and CT	lactoferrin (bLf)	[54]
EGFR	2'F-Py RNA	Hollow gold nanospheres	^{111}In	SPECT	2'F-Py RNA	[94]
Hepatocellular carcinoma cells	TLS11a	Gold nanoplatform	Chlorin e6	Fluorescence	PDT/PTT/AQ4N	[95]

CONCLUSION

As described in this chapter, aptamers are very powerful tools and have various applications as theranostic bionanomaterials for the diagnosis and treatment of cancer and other diseases. Their unique features make them an ideal noninvasive device for the diagnosis of cancer, even in early tumor stages. Additionally, comprehensive images and molecular information can be obtained by their *in vivo* imaging and without invasive biopsies. Such data will support clinical decision-making and provide new approaches and strategies for disease diagnosis and treatment. Importantly, as aptamers can utilize several ways of action, they are particularly attractive as tools for targeted therapy and can very well act as biosensors, biomarker detectors, targeted imaging tracers, and drug-delivery carriers. Due to their high performance in targeted inhibition, they can be considered as potential and competitive alternatives to antibodies.

However, some limitations and significant problems should be overcome, such as degradation in blood and high renal clearance. For RNA aptamers, chemical modifications of (3′-, 5′-) ends and (2′-) position provide resistance to exonucleases and endonucleases, respectively. In order to prolong the half-life of aptamer-circulation in the blood, conjugation with a variety of relatively high atomic mass species like polyethylene glycol (PEG) is a commonly used strategy. Recent development and advancement of aptamers will further present these molecules as an attractive platform for various translational applications. For the best use of aptamers, future directions should focus on aptamer-based biosensors and aptamer-based targeted drug-delivery systems, particularly systems for nucleoside analogue and oligonucleotide delivery for combined modality treatment approaches.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 7

Viral and Non-viral Nanoparticles for Gene Therapeutics

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Abstract: The recent accomplishment of the human genome and DNA discovery has led to the diagnosis of many diseases caused by imperfections in genes. These diseases involve gross disturbances in the number or arrangement of a person's chromosomes. Hence, gene therapy has become a promising new therapy for the treatment of somatic diseases, for example, malignant tumours [1], severe infectious diseases, such as AIDS [2], and many genetic disorders, including haemophilia and cystic fibrosis [3]. Gene therapy introduces a gene into human cells to replace, delete, or correct gene function to produce a therapeutic protein with the desired action. This adjustable gene can be used to cure any disease. In 1990, a gene therapy clinic was initiated to find treatment for severe combined immunodeficiency (SCID). However, the first success of gene therapy was not observed until 2000 when Cavazzana calvo *et al.* [4] reported a success using gene therapy for the treatment of SCID [4]. While it has been 30 years since the first gene therapy trial, gene therapy is still a high-risk treatment, and only a few drugs have been approved, such as Glybera®, Gendicine®, and Strimvelis®.

Keywords: Dendrimers, Gene therapy, Liposomes, Non-viral vectors, Viral vectors.

INTERNAL AND EXTERNAL BARRIERS TO GENE THERAPY

The delivery of genes into eukaryotic cells faces many barriers. It is estimated that the half-life of naked DNA is ten minutes following intravenous injection [5]. Many nucleases present in the extracellular matrix will rapidly degrade unprotected nucleic acid following systemic administration. Phagocytes, such as Kupffer cells in the liver, and resident macrophages in the spleen, are responsible for the clearance of DNA-loaded colloidal particles administered through blood circulation. In addition, poor penetration due to high hydrophilicity, hepatic first-

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pass metabolism, and a highly negatively charged phosphate backbone of DNA prevent its passage across negatively charged cellular membranes.

For this reason, the encapsulation of plasmid DNA in order to protect it and increase circulation time is a crucial step in developing gene therapy. In addition, the mechanism of gene therapy release from endosomes and lysosomes can significantly impact gene degradation in lysosomes; Fig. (**1**) shows a chart for the gene delivery pathway, an illustration of the gene pathway, and barriers to its delivery [6]. Macromolecules captured within the endosomes usually transform into digestive lysosomes unless some escape mechanisms are used to intercept this maturation process.

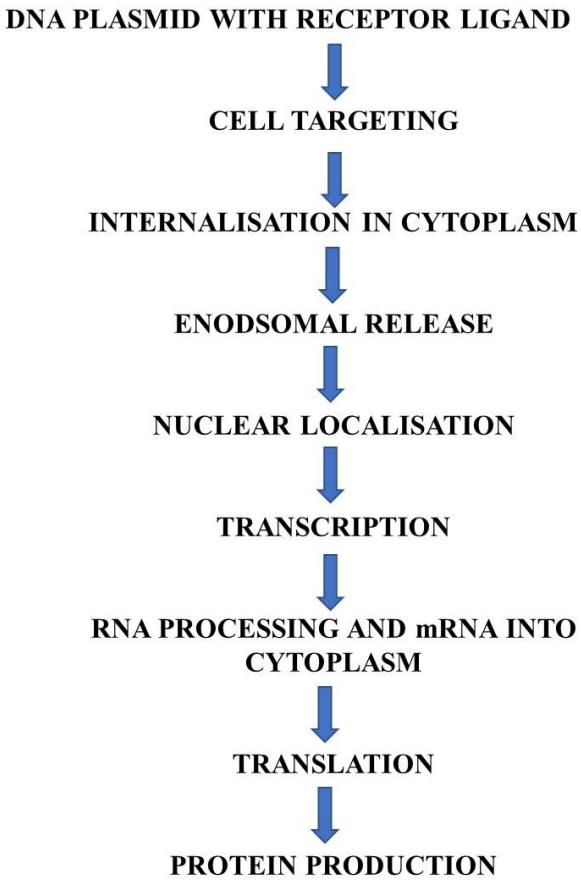

Fig. (1). A chart for the gene delivery pathway.

GENE DELIVERY METHODS

The gene delivery system is an initial phase in the achievement of gene therapy. Polynucleotide molecules (*e.g.*, plasmid DNA, RNA, and antisense oligonucleotides) are sensitive and hydrophilic, with a negatively charged phosphate backbone at physiological pH. Such physicochemical properties restrict their binding to and passive diffusion across lipophilic cell membranes. Moreover, they are rapidly degraded in biological fluids and do not accumulate in target tissues following systemic administration. In order to successfully transfect DNA into cells, DNA must be condensed and protected against nuclease degradation.

Additionally, the negative charge of DNA must be masked in order to allow entry through the negatively charged cell surface. After loading the genetic material to the vector, the vector must be delivered to the blood vessels and must be stable enough to avoid clearance by albumin due to their high surface charge. Next, the vector must pass through the epithelial tissues of the blood vessels and enter the target cell through the endocytosis process [7, 8]. Fig. (**2**) shows the most commonly used non-viral vectors in gene therapy.

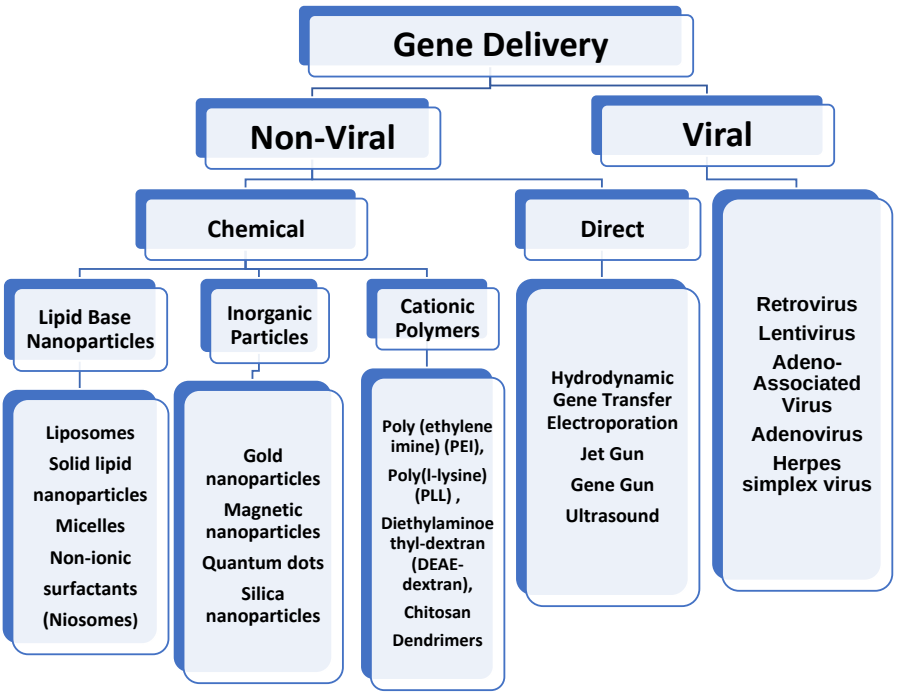

Fig. (2). The most commonly used non-viral vectors in gene therapy.

Gene Delivery Using Viral Vectors

Viral vectors have been used in most gene delivery clinical trials, accounting for more than 69% of the current clinical trials. Viruses are a powerful tool in vectoring gene products into cells. Viruses' structure utilizes the ability of the virus to inject their DNA inside host cells. In addition, virus structure will prevent the degradation of generic materials *via* the lysosome: adenoviruses, Adeno-associated viruses, Herpes simplex viruses. Furthermore, Retroviruses are the most common viral vectors which have been treated genetically to be non-pathogenic, and at the same time, they preserve their cellular infection. This can be attained *via* the coding regions of the viral genome that must be removed to stop the replication while preserving the sequence required for virus packing with the genes needed for expression within the host DNA to produce the proteins [9]. The deficient viral particles form the viral vectors. Each of the

DNA viruses can enter cells differently, such as endocytosis and fusion with the cell membrane [10]. The clathrin-mediated endocytic pathway is the most common pathway for adenoviruses to enter the cell, where acidification of the endosome causes the conformational changes in the adenovirus capsid leading to endosomal escape from lysosomal degradation [11]. Herpes simplex virus enters the cell by membrane fusion, resulting in the viral tegument as well as the DNA-containing capsid being deposited at the periphery of the cytoplasm, where it has been shown to localize with dynein [12].

Viral gene delivery is divided into the germline gene delivery systems and the somatic gene delivery systems. The germline gene delivery cannot be used for ethical reasons, and only the somatic cell alteration can currently be used. The somatic cell alteration has three subtypes (1) *in vivo* delivery, where the genetic materials transfer directly into the targeting tissue (2) *ex vivo* delivery the genetic materials implant into the targeting tissue bone marrow, cultivated, and manipulated *in vitro* then placed back into *in vivo* (3) *in situ* delivery system, the administration of the genetic material goes directly into the target tissue.

Viral vectors based on DNA for gene delivery are typically replicated by either host or virally encoded DNA polymerases. Viral vectors based on DNA are usually longer lasting and integrated into the genomes. DNA-based viral vectors are lentivirus, poxvirus, adenovirus, adeno-associated virus, retrovirus, human foamy virus (HFV), and herpes virus. Genomes of DNA viruses that infect animals range in size from less than 2 kb of single-stranded DNA to over 375 kb of double-stranded DNA [13]. RNA-based gene delivery is not permanent. Positive-sense RNA virus genomes formed first because infectious RNA could be transcribed directly from cDNA of the RNA genomes. This is followed by

employing negative RNA virus genomes as more complex approaches to provide RNA-dependent. This process will provide the RNA-dependent polymerase complexes with negative-strand RNA templates [14].

The main advantage (Table **1**) of viral vector systems is their ability to achieve high efficiency of transfection and high gene expression levels. However, their potential to induce immunogenicity and insertional mutagenesis have provoked a significant debate on their safety for gene therapy [15]. Moreover, immunogenicity danger remains due to the possibility of mutagenesis, and this could result in a malignant tumour by deleting the tumour expressing gene or switching on an oncogene. Unfortunately, in 2002/3, two children developed cancer after being treated with a retrovirus vector, and in 1999, one patient died due to using an adenovirus vector [16].

Table 1. The main advantages and disadvantages of the major viral vectors used in gene therapy, with their DNA incorporation type.

Viral Vector	DNA Incorporation Type	Advantage	Disadvantage	References
Retrovirus	Integrates with host chromatin	• Effective over long periods • Efficient transfection *ex vivo* • Low immune response in host	• Small, max 8kb insert size • Inefficient transfection *in vivo* • Relies on target cell mitosis • Safety concern	[17]
Lentivirus	Integrates with host chromatin	• Transfects proliferating and nonproliferating host and haemo stem cells • New generations are self-inactivating for safety	• Requires active transport into the cell, small max 8kb insert size • Safety concern • Immunodeficiency origins	[17]
Adeno-Associated Virus	Either host chromatin or chromosomal DNA	• Very good length of the expression, especially *in vivo* • Low immune response	• Safety problem owing to potential insertional mutagenesis • Small, max 4.5kb insert size • High immune response	[18]

(Table 1) cont.....

Viral Vector	DNA Incorporation Type	Advantage	Disadvantage	References
Adenovirus	Extra chromosomal DNA	• Highly efficient transfection *in vivo* and *ex vivo*. • Transfects proliferating and nonproliferating hosts.	• Repeat treatments ineffective due to strong immune response • Small, max 7.5kb insert size • Short expression Duration • Technologically • challenging	[19]
Herpes simplex virus	Extra chrolosomal DNA	• Very good length of expression especially *in vivo*. • Safe for use non-immunocompromised Patients.	• Difficult to produce in large quantities	[20]

Direct Injection of Plasmid DNA

These methods deliver the DNA directly into the target cell by physical method. Direct DNA delivery includes ballistic DNA injection, Electroporation, Gene Gun, and Ultrasound (Table **2**). These methods create temporary, weak points in the target cell membrane, allowing the genetic materials to enter the cell by diffusion. Direct injection of therapeutic genes into muscles or skin has been a handy tool in evaluating various aspects of DNA-based treatment. Different methods have been used, including Hydrodynamic Gene.

Table 2. Summary of the different methods for direct delivery of naked DNA, and the Table shows the advantages and disadvantages of each method.

Method	Mechanism	Advantage	Disadvantage	Reference
Jet Gun	Solution of DNA powered by gas and fired into the host cell.	Good for local treatment such as breast cancer and skin.	It can cause bleeding and bruising.	[21]
Hydrodynamic Gene Transfer	A high-pressure solution of DNA is pumped into the body through vein or catheter.	High transfection, up to 40%	Requires a very large volume	[22]
Electroporation	Electricity is used to alter the permeability of the cell wall and cause gene transfer	Safe and effective	Difficulty in accessing treatment sites with electrodes	[23]

(Table 2) cont.....

Method	Mechanism	Advantage	Disadvantage	Reference
Gene Gun	An inert gas is used to fire heavy metal particles coated with DNA at the target site and transfer DNA on impact	Good for muscle and tumor treatment, achieves higher expression rates	Difficulty in accessing treatment sites with electrodes	[24]
Ultrasound	Combined ultrasound energy and microbubbles to open cells membrane to plasmid DNA	Safe and efficient, good gene expression in vascular cells and muscles	Difficulty in accessing treatment sites with electrodes	[25]

Chemical Based Vectors

Synthetic vectors are chemicals that can condense DNA or RNA into nanoparticles with a size of less than 1μm to protect and mediate cellular entry. Cationic lipids and cationic polymers are studied as non-viral chemical-based gene delivery systems [26].

Lipid-Based Gene Delivery Carriers

Liposomes

Liposomes are formed by self-assembly of dissolved phospholipid molecules, each consisting of a hydrophilic head group attached to a hydrophobic tail [28]. The amphiphilic nature of the lipid molecules causes them to form bilayers spontaneously in aqueous environments. This results in a small, spherical structure in which the polar surface heads shield the non-polar interior against water. The presence of the positively charged amine facilitates binding with anions such as those found in DNA or RNA. Liposomes are attractive due to their flexibility regarding the size of DNA transported and their ability to carry DNA to various target cells.

Additionally, liposomes can protect DNA against enzymatic degradation and facilitate their cellular uptake and endosomal escape, leading to effective gene transfer. Cationic liposomes are the most studied liposomes for gene therapy, and their main components are cationic lipids. Cationic lipids are amphiphilic molecules; they share the typical structure of a positively charged hydrophilic head and a hydrophobic tail connected *via* a linker.

Cholesterol is an amphiphilic steroid molecule that plays a strategic role in liposome composition. Once cholesterol is added to the lipid, it orients its OH group towards the aqueous phase and the aliphatic chain towards the hydrocarbon

chain, thus restricting the movement of the carbons within the hydrocarbon chain [29]. This brings about changes in the fluidity and permeability of liposomes. The inclusion of cholesterol in liposomes has improved liposome rigidity to make them less leaky and increase liposome dynamic diameter and entrapment efficiency. The solution of cationic liposomes is usually formed with natural lipid helper and mixed with DNA, forming a positively charged complex known as a lipoplex. Most commonly used lipoplexes contain cationic lipid, cholesterol, and dioleoylphosphatidylethanolamine (DOPE). Helper lipids play a significant role in cationic lipid transfection activity by helping to convert the cationic lipid from a bilayer to a micellar structure, especially in lipids with single fatty chains.

In order to enhance cationic lipid release at the target cell, polymorphic cationic lipids have been developed. These are pH, temperature, and charge sensitive, where they undergo destabilization and acquire fusogenic properties under acidic conditions, releasing their content. The development of the polymorphic cationic lipids was proposed after it was reported that cancer cells and inflamed tissues have an acidic environment. Polymorphic cationic lipid usually consists of phosphatidylethanolamine (PE) with an amphiphilic polymer containing an acidic group, such as a carboxylic group which acts as a stabiliser in the presence of the compound at neutral acid [30]. The setback of cationic lipids in gene delivery may be blamed on the complexity of the transfection pathway, which is affected by the variation in the cationic lipid structure, such as the linker group, length of the hydrophobic tails, and the head group [31].

Due to the excess surface charge, the circulation half-life of cationic lipids in the blood is very short. Systemic elimination of cationic lipids occurs upon forming larger aggregates *via* their interactions with the negatively charged serum molecules or cellular components (primarily erythrocytes and platelets). The interaction between serum proteins and lipoplexes has affected the systemic delivery of cationic liposomes, which can dramatically lower the transfection efficiency. Binding serum proteins to cationic lipid/DNA complexes leads to structural re-organization, aggregation, and dissociation. As a result, there has been intense effort to develop cationic lipoplexes which efficiently transfer genes in the presence of serum. PEGylated components have been widely used to shield delivery vehicles from blood components [32].

Solid Lipid Nanoparticle (SLN)

Generally, solid lipid nanoparticles (SLN) are made from lipids that remain solid at room and body temperature. This includes triglycerides (*e.g.*, tristearin), partial glycerides (*e.g.*, Imwitor), fatty acids (*e.g.*, stearic acid), and steroids (*e.g.*, cholesterol), which are particles with a size range from 50 – 1000 nm. The main

advantage of SLN is that it is made from physiological lipid, which decreases the danger of acute and chronic toxicity.

Non-ionic Surfactant Vesicles (Niosomes)

Niosomes are colloidal particles formed from the self-assembly of non-ionic surfactants in an aqueous medium, resulting in closed bilayer structures and also known as Non-ionic surfactant vesicles. Niosomes are mainly composed of non-ionic surfactants such as alkyl or dialkyl polyglycerol ethers, with or without cholesterol. Non-ionic surfactants most commonly used in niosome formulations are tweens and Span. These surfactants are amphiphilic molecules containing both hydrophilic heads and hydrophobic tails. Niosomes can deliver hydrophilic such as DNA or RNA Hydrophilic drugs are condensed in the centre core, whereas hydrophobic drugs are bound to the outer surface.

Lipid Micelles

Micelles are prepared from amphiphilic molecules, which contain both hydrophilic heads and hydrophobic tails, with the lipophilic tails forming the inner core and the hydrophilic heads exposed toward the aqueous environment. Depending on their composition, micelles can vary in shape from spherical to rod-like or ellipsoidal structures. Phospholipids as a hydrophobic block of copolymers to prepare lipid-core micelles have increased micelle stability. This increased instability is due to the hydrophobic interaction between double acyl chains of phospholipids, allowing hydrophobic drugs to be solubilized [33].

Cationic Polymers

Many cationic polymers have been investigated in gene therapy. Once the cationic polymer is mixed with DNA, it forms a complex called a polyplex which is more stable than a lipoplex.

Poly(l-lysine) (PLL)

Poly(l-lysine) (PLL) is one of the most investigated polymers in gene therapy. They are polypeptides with amino-acid lysine as a repeating unit and are biodegradable. Moreover, the amine groups can enhance their buffering capacities leading to endosomal or lysosomal rupture and escape into the cytoplasm *via* a proton sponge effect [34]. PLL has been widely used as a non-viral gene carrier since the formation of polyelectrolyte complexes between PLL and DNA.

However, the lack of rapid PLL/DNA complexes released from endosomes resulted in low transfection efficiency. In addition, PLL with a high molecule weight (Mw) is more prone to aggregation and precipitation, resulting in high cell toxicity. The strategy of "PEGylation" (formation of PEG-PLL conjugates to improve biocompatibility) has been widely used in gene delivery. PLL-PEG usually synthesised by ring-opening polymerization of N-carboxy anhydride (NCA) and derivatized lysine monomers. This reduced surface zeta potential and cell toxicity with a size of just over 100 nm. As potential cystic fibrosis and ocular degenerative disease, such polyplexes are being investigated in phase I and phase II clinical trials [35].

Poly (Ethylene Imine) (PEI)

Poly (ethylene imine) exists in either branched or linear structures. PEI contains primary (25%), secondary (50%), and tertiary amines (25%), of which two-thirds of the amines are protonated at physiological pH. The non-protonated amines in the PEI exert the so-called proton sponge effect, which effectively stops the acidification of the endosomal pH. This protects the DNA from degradation in the endosomal compartment during the maturation of the endosome to a lysosome, which facilitates the intracellular trafficking of DNA. In the same way as PLL, PEI, with its high molecular weight, forms a highly condensed particle when mixed with DNA. However, this brings high toxicity (greater than 25,000 Da). Many studies have shown that linear PEIs produce higher transfection efficiencies and lower cytotoxicity than branched PEIs [36].

Diethylaminoethyl-dextran (DEAE-dextran)

Dextran is a polysaccharide composed of glucose units, and DEAE-Dextran is formed when the coupling between diethyl aminoethyl groups leads to amidation of the dextran chain and subsequent protonation. The strong attraction between DNA or RNA and DEAE-dextran caused by the cationic property of the DEAE-dextran has been found to change its adsorbing power for nucleic acids through pH and ion strength [37]. Cationic polymers such as DEAE-dextran are self-assembled with DNA to form complex polyplexes with a high affinity for the cell membrane [38]. Siewert *et al*. [39] reported that DEAE-dextran as cationic polymers at an N/P ratio of more than 2 resulted in positive zeta potential, leading to increased encapsulation and transfection efficiency of mRNA [39].

Dendrimers

Dendrimers consist of a central core molecule that acts as the roots of several branches. These highly branched three-dimensional macromolecules became the focus of research in drug and gene delivery. The fundamental properties that proved the use of these materials in drug and gene delivery are defined by the high density of terminal groups contribute to the molecules' surface characteristics, and this will provide multiple attachment sites for drugs or genes, the molecular volume will determine their ability to sequester other molecules within their core.

Chitosan

The cationic polysaccharide chitosan is one of the most widely studied non-viral vectors. Positively charged chitosan can be complexed with negatively charged DNA without the need for sonication, which reduces the risk of DNA damage. Although it has shown high nucleic acid binding, transfection efficiency is still very low. In order to improve chitosan transfection efficiency, novel chitosan derivatives have been synthesised, such as aminoethyl- chitin (ABC), thiolated-chitosan, chitosan methoxy poly-ethylene glycol- cholesterol (LCP- Ch), and low molecular weight alkylated chitosan [40].

Inorganic Particles

Inorganic nanoparticles can be engineered to different sizes, shapes, and porosity to condense and protect genetic materials from degradation and escape from the reticuloendothelial system.

Gold Nanoparticles

Gold nanoparticles (GNP) have been primarily used in clinical diagnostic methods, while currently, it is showing great promise *in vivo* as a therapeutic agent. The unique properties of GNPs, such as a wide range of core sizes from 1nm to 150 nm, high stability in saline solutions, and have a high affinity for biomolecules, are desirable as non-viral vectors for DNA delivery. Gold nanoparticles are synthesized by the chemical reduction of chloroauric acid (HAuCl4) using reducing agents [41]. The ease of functionality and tailoring surface of GNPs can be used to modify GNPs to gain accessibility and effectiveness to target cancerous tissues. Because of their demonstrated stability and enhanced binding properties, this particular type of GNP could efficiently scavenge intracellular DNA or RNA.

Magnetic Nanoparticles (MNPs)

Christian Plank and co-workers first developed magnetic nanoparticles in 2002 for gene transfer in cell cultures and *in vivo* [42]. MNP-naked DNA complexes are based on the association of MNPs with gene vectors in a process called magnetoreception, which is used to enhance gene transfer in the presence of a magnetic field [43]. The most commonly used materials for magnetic drug delivery contain metals or metal oxide nanoparticles, such as superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs consist of an iron oxide core and are usually coated with organic materials such as fatty acids or polymers to improve colloidal stability and prevent separation into particles and carrier medium [44]. Liposomes are also frequently developed and applied, for instance, chitosan-coated magnetic nanoparticles after encapsulation inside polyethyleneglycol (PEG) related liposomes or magnetic nanoparticle-supported lipid bilayers constructed around the core-shell Fe_3O_4–SiO_2 nanoparticles [45].

Quantum Dots (QDs)

QDs are bright photostable CdSe/ZnS fluorescent nanocrystals that exhibit tunable emission properties for a wide range of colors. Solvothermal pyrolysis methods prepare QDs. The initial surface ligands of QDs are typically fatty acids, such as oleic acid, which can be accumulated in adipocytes and nonadipocytes. In order to apply them in an aqueous phase, the hydrophobic surface ligands need to be exchanged by foreign ligands or be encapsulated by amphiphilic macromolecules [46]. The main advantage of QDs is their lack of toxicity to normal cell physiology [47].

Moreover, QDs possess excellent photostability, high photoluminescence quantum yield, are brighter than most conventional fluorescent dyes by 10-fold. QDs have been significantly more straightforward to detect than green fluorescent protein (GFP) and have a wide range of excitation spectrum and a long lifetime of fluorescence [48]. QDs have unique fluorescent properties, so there is no need for chemical labeling to track the investigated plasmid's expression.

Mesoporous Silica Nanoparticles (MSNs)

MSNs have been used in controllable drug delivery, gene transport, gene expression, biomarking, biosignal probing, imaging agents, and drug delivery

vehicles. MSNs have a honeycomb-like structure with an active surface that enables surface modification properties and links therapeutic molecules [49]. MSNs have become a promising drug vehicle due to their superior structural properties, such as high surface area, large pore volume, tunable pore diameter, and narrow pore size distribution [50].

CELL TARGETING

There are two different types of targeting, active targeting, and passive targeting.

Active Targeting

Active targeting is achieved through surface modification of the nanoparticles with cell-specific biorecognition molecules. Nanoparticles will recognise and bind to overexpressed receptors on the surface of the targeted cells. Most commonly, nanoparticle receptors for active cell targeting include TF, folate, epidermal growth factor (EGFR), cell surface glycoproteins, single-stranded DNA (ssDNA), and RNA aptamers.

Passive Targeting

Passive targeting is achieved by utilising the unique and distinct pathophysiology of tumor cells and the physicochemical properties of the nanoparticles [51]. The accumulation of lipid-based nanoparticles on the target cell results from the enhanced permeability and retention (EPR) phenomenon. EPR phenomenon results from the leaky vasculature of tumor cells, which allows macromolecules with a diameter of ≥600 nm to extravasate into the neoplastic tissues. This is caused by the increased demand for oxygen and nutrients from rapidly growing malignant cells, which cause dilation of numerous pores and wide gap junctions between endothelial cells [52]. The smooth muscle layer of normal blood vessels mediates a vasogenic response to maintain a constant blood flow to an organ. However, these mechanisms are missing in the microvasculature of neoplastic tissues, causing a state of permanent vasodilation.

POTENTIAL TOXICITY OF NANOPARTICLES

Although there are many advantages of nanoparticles' application, as discussed in the previous sections, the handling of these nanoparticles during the preparation, formulation, and processing should be with cautions because some new studies have demonstrated that nanoparticles may lead to severe health problems after

their exposure into the body *via* inhalation, digestion and skin contact. Their toxic effects (because they can form reactive species and cause cytotoxicity, neurotoxicity, *etc*.) are based on nanoparticle characteristics, such as size, zeta potential, and shape [53].

CONCLUSION

Gene therapy has become a promising new therapy for treating somatic disease, severe infectious diseases, and many genetic disorders. The fact that many nucleases present in the extracellular matrix will rapidly degrade unprotected nucleic acid. In addition, poor penetration due to the highly negatively charged phosphate backbone of DNA prevents its passage across negatively charged cellular membranes. For this reason, the encapsulation of plasmid DNA in order to protect it and increase circulation time is a crucial step in developing gene therapy. Numerous viral and nonviral gene delivery systems have been developed. Viral vectors guarantee high transduction efficiency of delivery due to their inherent ability to introduce exogenous genetic material into the cells. Nevertheless, immunogenicity and mutagenicity associated with viral delivery have limited the widespread use of viral delivery systems. Despite the low cytotoxicity and low immunogenicity of nonviral-based vectors, low transfection efficiency compared to viral vectors is still the main obstacle to their development.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

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CHAPTER 8

Conclusion, Outlook, and Prospects: Bionanomaterials in Clinical Utilization

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Abstract: Nanomaterials have contributed to significant advancements in the realms of biotechnology and medicine. A holistic examination of the different biocompatible nanocomposites is discussed in this chapter. Their compatibility with state-of-the-art engineering techniques, such as additive manufacturing to design practical surgical implants, is also discussed. The importance and potential of nanocomposites and manufacturing processes in implantable medical device industries are also thoroughly considered. Nanomaterials' unique characteristics contrast with their large counterparts, such as high surfaces, reactivity, and reproducibility. Their incorporation in matrices has shown that the resultant composites' mechanical, chemical, and physical properties can be improved.

Consequently, a wide variety of technical technologies, such as energy products, biomedical applications, micro-electrical equipment *etc*., have been intensively researched. Furthermore, the foundation for many new medicines and surgical instruments, including nanorobots, has been built on nanobiotechnology. It has been utilized in almost every medical sector, and its usage in the treatment of different diseases, such as cancer, neurobiology, cardiovascular disorders, joint and bone disorders, eye diseases, and infectious diseases, has been evident through different studies. Nanobiotechnology can promote diagnostics and the advancement of customized medicine, *i.e*., prescribing unique therapeutics that are tailored to an individual's needs. Many advances have already begun, and a definite effect on medicine practice will be felt in a decade.

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INTRODUCTION

In recent years, nanotechnology and nanobiology have gained a significant boost. The latest growth in the applications of polymer composites is gathering momentum worldwide. Biotechnology has been revolutionized as an assertive discipline utilizing bionanotechnological research and development. Surface functionalization at nano-levels is a crucial aspect of bionanotechnology, in which several entities have already been exploited. Bio-nano substances can be used for diagnostic and therapeutic purposes by using polymers, bacteria, nucleic acids, antibodies, or other proteins [1]. Bionanotechnological advancement has made early diagnosis and treatment more possible. This makes perfect sense since more lifetimes will be spared if doctors can promptly diagnose lethal disorders and diseases. Bionanotechnology is referred to as an inspirational catalyst in a nanotechnology discipline. Speculation is being made that progress will be made more and more as biotechnology integrates [2, 3].

The extraordinary features and establishment of advanced polymer composites with established environmental analytical techniques contribute to the tremendous success of boosting the environment research era. Over the last few decades, nanotechnology's development has been fueled by the need for new nanomaterials with unmatched properties from their counterpart synthetic nanoparticles. Such properties include biodegradability, biocompatibility, and unmatched uniformity in structure and composition, with the amenability of chemical and biological modification to impart new and novel properties that make biomaterials ideal nanoparticles [4]. Newly developed nanomaterials showed promising medical, pharmaceutical, environmental, and energy applications. This chapter highlights different types of nature-inspired and biosynthesized nanomaterials and their green synthesis methods and some of their emerging applications, especially in nanomedicine, cosmetics, drug delivery, molecular imaging, and catalytic precursors. The chapter covers different types of bionanomaterials (*e.g.*, viruses, protein cages, phages) and highlights their unique properties and potential applications [5, 6].

Treating certain illnesses is a big challenge in delivering treatment compounds to the target site. A typical drug application may be constrained in potency, poor biodistribution, and lack of selectivity. Nanocages have been shown to be preferentially clustered in tumors or inflammatory sites due to antigen sampling due to the improved vasculature permeability and retention (EPR) effect. Present means of treating the bulk of solid tumors are surgery and chemotherapy or

radiation treatment. However, today's therapy destroys healthy tissue and induces unjustified patient toxicity in its general and systematic implementation. Nanoparticular delivery systems in various biomedical applications have been used in recent times. The attributes of these products are biocompatible, well-defined, and readily functional materials. Mainly due to these materials' properties, nanomaterials demonstrate high differentials in the cell or tissue-specific targeting and absorption ability. Besides, before hitting the target, the drug molecule on the nanocarrier is shielded from harsh conditions. Contrary to standard drug delivery, a delayed and regulated release of drugs can be accomplished with nanomaterials. Therefore, nanomedicine represents a revolutionary field of tremendous treatment potential by combining intelligent nanoparticles with a wide variety of functions [7].

ADVANTAGES OF NANOBIOTECHNOLOGY

The pathophysiology and the anatomic modifications of diseased or inflamed tissue have paved various ways for producing targeted nanotechnology products. In the following respects, this growth is advantageous: 1. The pathophysiological properties of diseased tissues may be used to target drugs; 2. Different nanoproducts can accumulate at concentrations above standard medicinal products; 3. Enhanced vascular permeability in tumors and impaired lymph drainage enhance tumors or inflamed tissue effects of nanosystems by improved dissemination and retention. 4. The potential of the nanosystems to locate inflammatory tissues is selective. 5. Nanoparticles may be used to supply/transport medicines to the brain that overcome the blood and brain barrier (meninges). 6. Nanoparticle loading modifies the distribution of cells and tissues and contributes to the targeted delivery of biologically active substances to increase drug production and mitigate drug toxicity [8, 9].

Applications of Nanobiotechnology in the Biomedical Field

Several therapeutic uses of nanobiotechnology are currently being studied on various topics, such as cancer detection, target drug delivery, and molecular imaging. Clinical trials are now underway on several new, exciting drugs. These innovative applications of biological systems will undoubtedly be the basis for future diagnosis, care, and disease prevention. The optimum utilization over alteration and functionalization of certain noncarrier is a significant and critical aspect to be taken into consideration when NPs are used as drug delivery system; two fundamental principles, surface alteration and targeting, are discussed here; these two concepts, nevertheless, can in some cases be used to the same degree. Altering processes are implemented for targeting purposes, including the defense

of nanocarrier and the diagnostic/therapeutic aggregate loaded against the immune system. Surface shift depends on the NPs portion, size, and surface [10]. A significant benefit of nanosystems is that they are being built from various natural or synthetic materials; hence, various alteration methods have been employed. For instance, they are used in protein-based vehicles to act at three different internal, external, and inter-subunit subatomic positions or connections. Chemical and genetic engineering of protein cages has been used to alter the sequence of amino acids of surface-accessible residue protein nanostructures [11 - 14]. Alterations may also be achieved by converting certain amino acids into others or using chemical reactions to change current amino acids. The molecular versatility can monitor the surface load, enhance particle stability, and enclose medicinal products. On the other hand, various methods for modifying inorganic nanosystems, such as gold and silica nanosystems, are introduced [15].

Diverse genetic/chemical alteration methods have been proposed for virus-like particulates (VLP) derived nanosystems to provide benefits, such as cargo packing hydrophobic cargos, imagery/therapeutics connection, effective gene and drug entrapment and distribution, the stimulus-driven release of medications, various cancers targeting, *etc*. Several charges, including nuclear acids, imaging agents, anticancer medications, *etc*., have been encapsidated through chemical conjugation or genetic injection [15]. Viruses are infectious natural materials-based agents, are protein-covered by foreign proteins, and replicated by live cells. Viral capsid proteins can construct the hollow spherical structure, such as caged platforms, with single-layer walls known as icosahedral-like particles (VLP), as shown in Fig. (**1**). A range of medical applications as a relatively new kind of nanomaterials include platforms for delivering cargo therapeutics. The latest research has explored virus-inspired distribution mechanisms. VLPs are not found contagious without nucleic acids. After releasing the nucleic acids, pH-mediated swelling and alkaline hydrolysis intact viral NPs can be converted into VLP. Viral NP protein subunits can be disassembled into VLP [16 - 18].

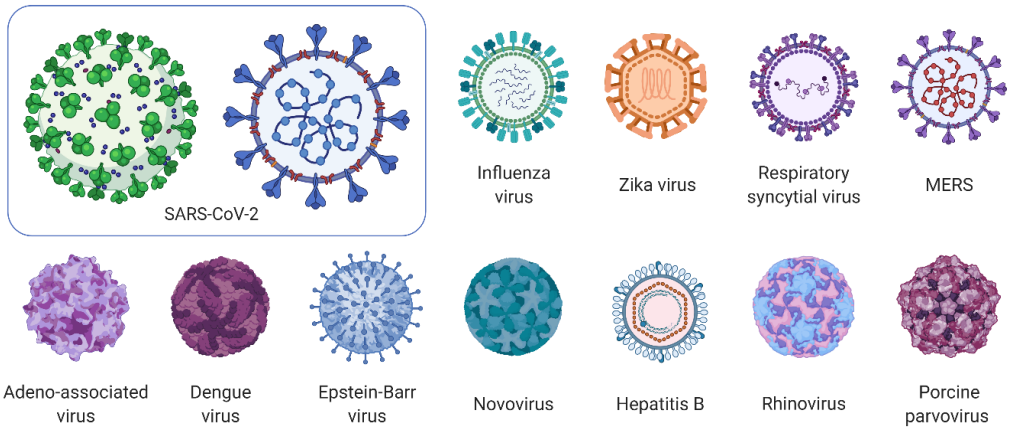

Fig. (1). Schematic illustration of viral arrays displaying different icosahedron morphologies.

VLPs, such as nanosystems based on CCMV, have shown the abnormality and morphological changes caused by protein-protein and protein-RNA interaction to respond to pH, temperature, and ion resistance. These nanosystems could be ideal for transporting different cargoes [19]. VLPs have several essential features that make them suitable for the distribution of drugs, including (1) being very stable and self-assembled in the range of 10 nm to 200 nm; (2) simple, wide-scale purification; (3) all particles are the same in viruses and VLPs for each form, which can be called monodisperse NPs; and (4) they display strong geo-purification; VLPs have several significant functions. Nucleic acid (DNA, RNA) can be delivered and transferred by VLP-based NCs in various conditions, such as low or elevated pHs, hot humidity, and target immune systems [20]. The viral capsid is usually a VLP consisting of three chemically distinct interfaces: the inside surface, the outside surface, and the interface between subunits. With well-engineered bio-conjugation reactions, viral capsids' exterior surface can be controlled employing peptides, fluorescent dyes, polymers, carbohydrates, and oligonucleotides. Besides, genetic modification strategies will alter the capsid proteins' amino acid sequence, thereby offering many possibilities [21].

A variety of agents can be used to alter or run ferritin-based nanosystems to increase cancer targeting by motive targeting, enhance cargo loading efficiency, allow simultaneous diagnosis and treatment, and provide a “stealth effect” that enhances medium-term therapeutic cargo usage [22, 23]. The earlier studies have been focused on altering genetic/chemical methods of small heat shock proteins (sHSPs) with findings, such as increased load supply and cell targeting [12, 24].

Diagnostic applications: Modern diagnosis methods for certain disorders rely on

obvious signs being manifested before doctors can understand that a particular illness affects the patient. However, medication may be less likely to succeed where these effects occur. The quicker a condition is discovered, the more excellent the opportunity for a cure. Diseases should optimally be detected and cured due to symptoms. Diagnostics of nuclear acid will play an essential role in this process. It allows the diagnosis of infections and diseases and makes successful therapy more workable in the asymptomatic early stage of disease development. Present technologies, such as polymerase chain reaction (PCR), contribute to such research and tools, but nanotechnology extends existing options to improve sensitivity, performance, and cost-saving [14].

Various current/conventional clinical trials show that an organism is causing disease by observing the binding of a particular antibody to the disease's target. Traditionally, such experiments are done using fluorescence or electronic microscopy to bind the antibodies with inorganic/organic dyes and visualize signals in the samples, as shown in Fig. (**2**). Dyes also, however, restrict the detection processes' precision and practicality. Nanobiotechnology uses semiconductor nanocrystals to give a solution (also referred to as “quantum dots”). These limited samples can tolerate much more excitement periods and light pollution than the typical ones. The biological phenotype of species in stable and diseased states is dependent on protein products, and their functionality is more significant. Proteomics is essential in disease diagnosis and pharmaceutical goods, where drugs can change signals. Protein chips may be handled with a small modular protein portion or chemical group that may explicitly be bound to proteins possessing a particular structural or biochemical motif. Agilent, Inc. and NanoInk, Inc. are currently two companies active in this field. Agilent uses non-contact ink-jet printing to print oligos and entire cDNAs on the nanoglass panes.

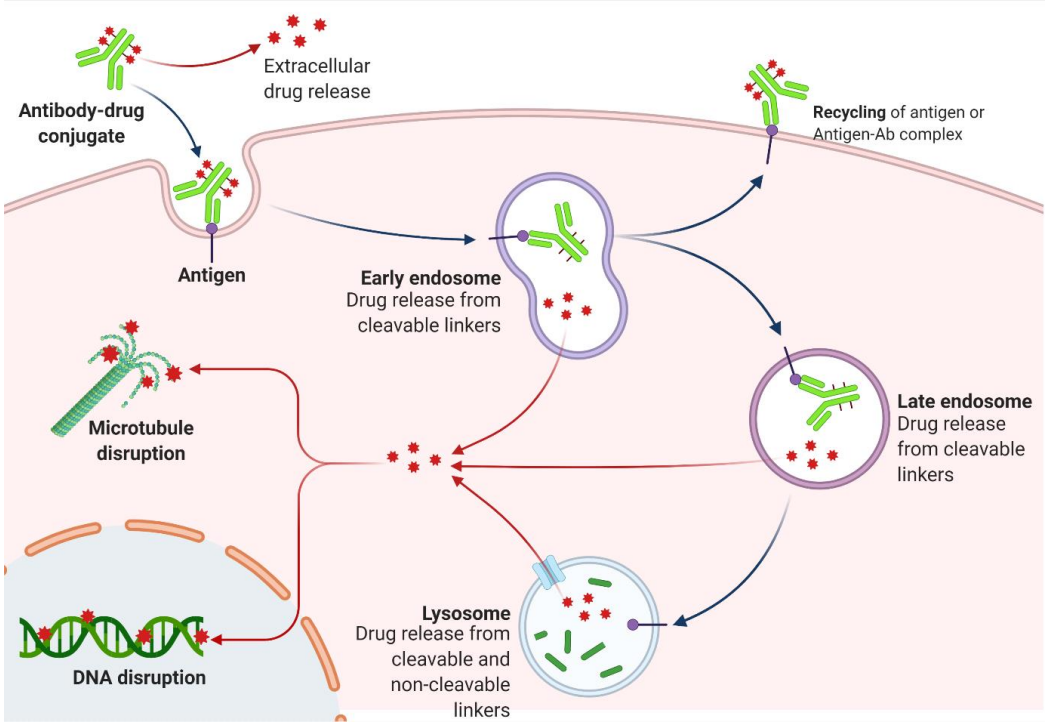

Fig. (2). Schematic illustration of the use of antibodies as an example of biological nanomaterials for the selective targeting of therapeutic agents to the targeted cells.

Imaging intracellularly by marking target molecules with quantum dots (QDs) or synthetic chromophores (such as fluorescent proteins) may be made possible, which promote direct in-cell signaling complex investigation by optical techniques *i.e*. Microscopy or imagery correlation with confocal fluorescence. Nanoparticles may be administered as therapeutics at specific places, even areas not readily accessible by traditional medicines [25]. *E.g*., suppose a nanoparticle can be chemically associated with a therapeutic. In that case, it can be directed by radiography and magnetic signals to the disease site or infection. These nanotropic substances can also be "released" when specific molecules exist, or external triggers (*e.g*., infrared heat) are present. Around the same time, the adequate dose necessary to treat the patient will be minimized by avoiding potent medication's adverse side effects. The release can be regulated even more accurately than ever by encapsulating drugs in nanosized materials (organic dendrimers, hollow polymer capsules, and nanoshells). Pharmaceuticals are designed for treatment and imaging uses with a therapeutic payload (radiation, chemotherapy, or gene treatment). Due to low bioavailability, many agents cannot be delivered orally and can now be used in nanotechnology. Nanoformulations

provide immunity against degradation or denaturation susceptible agents when subjected to intense photo-hypothesis and extend half-life by increasing the formulation's biodiversity conservation; some applications are depicted in Fig. (**3**). The provision of vaccine antigens is also a broad application of nanotechnology. Recent progress in encapsulating and creating effective animal modeling has shown that microparticles and nanoparticles may improve immune systems.

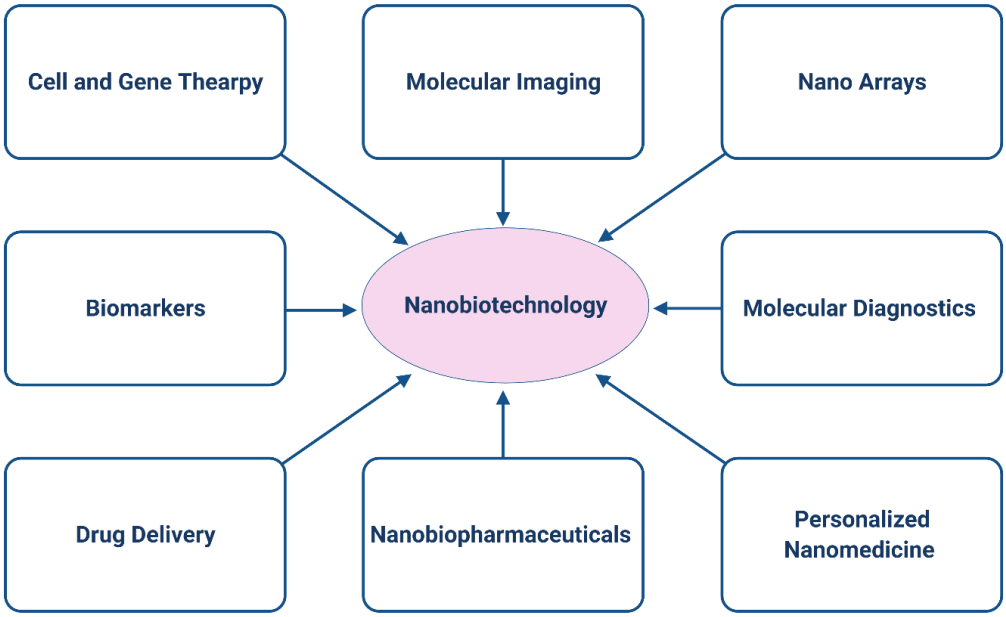

Fig. (3). Schematic illustration of nanobiotechnology relationship with nanomedicine and other biotechnologies.

Present gene therapy programs could return to wild types and are prone to inherent difficulties of successful pharmaceutical production and growth. Often troublesome is the possible viral vector immunogenicity involved in the genetic transfer. Human gene therapy nanotechnology tools have been tested, and plasmid DNA transportation nonviral nanoparticles (usually 50-500 nm in size) were defined to overcome this issue. Thus, it appears beneficial to restore or substitute damaged genes in human beings if fewer immunogenically nanosized transporters are used to replace contested virus vectors.

The medical industry will continue to choose optical and colorimetric detections despite the benefits of magnetic detections. For example, Nanosphere (Northbrook, Illinois) is a company that developed techniques that enable physicians to diagnose the genetic makeup of organic samples optically. The

source of an easily read analysis for the identity of any given genetic sequence is tiny gold particles encrusted with small fragments of DNA. As the sequence of questions in specimens is linked to complementary extensions of DNA on several nanospheres, it forms a dense network of transparent gold spheres. It makes it easier to detect pathogens and has shown promise of success for identifying anthrax, which gives a much greater sensitivity than commonly used tests.

Biopharmaceuticals

Pharmaceutical formulations need to be improved by developing faster, cheaper, and less toxic preparations and therapies. The expertise of nanomaterials which has already developed innovations in the advanced distribution of oral formulations, injectable drugs, and transdermal and topic-level delivery formulations, can be faced with this requirement. The purpose of drug delivery would be to increase the availability and efficiency of therapies in the designated target and reduce toxicity and costs with a high payload. Nanobiotechnologies can produce disease medicines not targeted by traditional pharmaceuticals. Traditionally the pharmaceutical industry focuses on producing medicines to treat a given universe of approximately five hundred reported diseases. However, about 70% to 80% of new drug product applicants fail, and these errors are often found late in the development period, with millions of dollars lost in R&D funding. Smaller firms that cannot recruit hundreds of organic chemists to synthesize and analyze thousands of compounds will benefit from nanoscale techniques to manufacture medicines. Nanobiotechnology allows targets, molecules, and atoms to be mechanically handled on solid substrates by adding them to biomembranes and checking where chemical reactions occur in a rapid, material-free process (reagents and solutions). This advancement would minimize drug exploration costs, including a wide variety of compounds, and promote drug development. Potentia Pharmaceuticals is an early organization seeking to simplify the process of production by using nanotechnologies.

PROSPECTS OF NANOBIOTECHNOLOGY

The effects of nanobiotechnology for the future are widely debated. It will build various new materials and valuable equipment in the medical, electrical, biomaterials, and energy generation fields and could be applied. However, this strategy poses numerous issues as any emerging technology, including the toxicity and influence of nanomaterials on the environment, the possible impacts on the international economy, and the uncertainty regarding various doomsday scenarios. The dispute between activists and governments on whether special nanobiotechnology laws were needed was responsible for these concerns. This

technology offers enormous promise for the future, despite various conflicts. This can lead to advances in biotechnology, ranging from drugs and gene therapy to molecular imaging, biomarkers, and biosensors [1, 10]. This can lead to innovation. Today's key research goal is one of these applications: selective opioid therapy and early detection and prevention strategies of illness. In clinical diagnosis and R&D, two forms of medical applications already exist. Imaging applications like quantum dot technology are now approved, and wireless tissue surveillance applications will be in operation shortly. The next significant contribution is producing exact and responsive protein and DNA detection methods. From 2015 to 2020, we were already beginning to see drugs evaluated on the market in academics and government labs. Spacious cell isolation and molecular filtration applications can then fulfill the demand. Certain drugs should be marketed or in advanced clinical trials, as presented in Table **1**.

Table 1. Clinically available nanomaterials for drug delivery and approved clinical applications in drug delivery, and molecular imaging.

Nanoparticles	Nanomaterials Type/Drug	Approved Clinical Applications	References
DaunoXome	Non-PEGylated Liposomal daunorubicin	HIV-associated Kaposi's sarcoma	[26]
Myocet	Non-PEGylated Liposomal doxorubicin	Treatment of metastatic breast cancer	[27]
Abraxane	Albumin-particle bound paclitaxel	Advanced non-small cell lung cancer	[28]
Doxil	PEGylated liposomal doxorubicin	Ovarian cancer, Multiple myeloma, Kaposi's sarcoma	[29]
Marqibo	PEGylated Liposomal mifamurtide	Philadelphia chromosome-negative acute lymphoblastic leukemia	[30]
MEPACT	PEGylated Liposomal mifamurtide	Treatment for osteosarcoma	[31]
Onivyde	PEGylated Liposomal irinotecan	Metastatic pancreatic cancer	[32]
Diprivan	Liposomal propofol	Induction and maintenance of sedation or anesthesia	[33]
AmBisome	Liposomal amphotericin B	Preventing or treating invasive fungal infections	[34]
Resovist	Iron carboxydextran colloid	Imaging of liver lesions	[35]
Feridex I.V.	Iron dextran colloid	No current studies	[36]

(Table 1) cont.....

Nanoparticles	Nanomaterials Type/Drug	Approved Clinical Applications	References
Ferumoxtran-10/Combidex/Sinerem	Iron dextran colloid	Imaging lymph node metastases	[37]
SonoVue	Phospholipid stabilized microbubble	Ultrasound enhancement for liver neoplasms, prostate or breast or pancreatic cancer	[38]
Optison	Human serum albumin stabilized perflutren microspheres	Ultrasound enhancement for lymph node, renal cell carcinoma, myocardial infarction *etc*	[39]
Inflexal V	Liposome with trivalent-*influenza*	Safety and immunogenicity of *influenza* vaccine	[40]
Epaxal	Liposome with hepatitis A virus	Safety and immunogenicity of hepatitis A vaccine	[41]
Monofer	10% Iron isomaltoside 1000 colloid	Iron deficient anemia	[42]
Diafer	5% Iron isomaltoside 1000 colloid	Iron deficient anemia	[43]
Injectafter/Ferinject	Iron carboxymaltose colloid	Iron deficient anemia	[44]
Definity	Perflutren lipid microspheres	Ultrasound contrast agent	[45]
Feraheme	Iron polyglucose sorbitol carboxymethyl ether	Iron deficiency in patients with chronic kidney disease	[46]
Venofer	Iron sucrose colloid	Iron replacement for anemia treatment in patients with chronic kidney disease	[47]
DexFerrum/DexIron	Iron dextran colloid	Iron deficient anemia	[48]
CosmoFer/INFeD/Ferrisat	Iron dextran colloid	Iron deficient anemia	[49]

For example, NanoSystems or American Pharmaceutical Collaborators have developed drug delivery systems that measure cyclophosphamide encapsulation, a cancer drug in a paclitaxel nanopolymer. A decade or so from the industry is the bulk of medical instruments and therapeutics. Thus, both the handling of drugs and systems involve complex technological infrastructures, such as nanotechnology, and difficult regulatory management. Constant development in nanomedicine has opened up its possibilities in various medical disciplines. The future use of diagnostic and regenerative medicine is being researched now. Diagnosis would make it easier to diagnose diseasing cells, likely at the point of a single ill cell, thus allowing diseased cells to be healed quickly before dispersing

into other areas of the body. People with serious injuries or damaged organ functions may also benefit from nanomedicine.

ADVANTAGES OF NANOBIOTECHNOLOGY

Different target materials can be developed through pathophysiological conditions and structural modifications of tissues' disease or inflammation. Throughout the following aspects, such development is benefits are appeared as; The pathophysiological characteristics of the diseased tissues may be used to accomplish the drug targeting. Several nanoproducts may have a greater intensity than typical pharmaceutical products, enhanced permeability of the vascular system, impaired lymphatic drainage in the tumor, and increased tissue dissemination and retention. In inflammatory tissues, nanosystems have selectively located energy. Nanoparticles can be used successfully to supply/transport-related medications to the brain that overcome the blood-brain barrier (meninges). Loading nanoparticles modify the distribution of cells and tissues and lead to a more selective provision of biologically active substances that improve drug effectiveness.

CHALLENGES FOR NANOBIOTECHNOLOGY

There are five key obstacles,such as: designing tools for testing susceptibility in the air and water to engineered nanomaterials, as shown in Fig. (**4**). It is reasonably known that there could be some potentially harmful consequences of humans and animals proximity to the water, which is potentially nano-contaminated. The problem is becoming more challenging in more complex matrices, such as food. The next challenge is to improve the relevant methods of detecting and assessing engineered nanomaterials toxicity in five to 15 years. Once again, it will be inevitable to suggest models for estimating these nanomaterials human and environmental impact. Another task will be to build reverse systems to assess engineered nanomaterials accurate health and environmental effects over the life cycle problem. Next, designing the tools to determine risks to human health and the environment correctly is another big obstacle. Nanobiotechnology marketing problems include the complexity of innovation feasibility, scalability, financing, finite money, patience, *etc*. A substantial majority of the organization acknowledges that nanotechnology has tremendous potential for designing innovative technologies and refining existing ones. The need for new legislation poses fundamental concerns in a new potentially revolutionary technology, such as nanotechnology. Global authorities should identify potential risks and have an acceptable regulatory answer to their widespread usage.

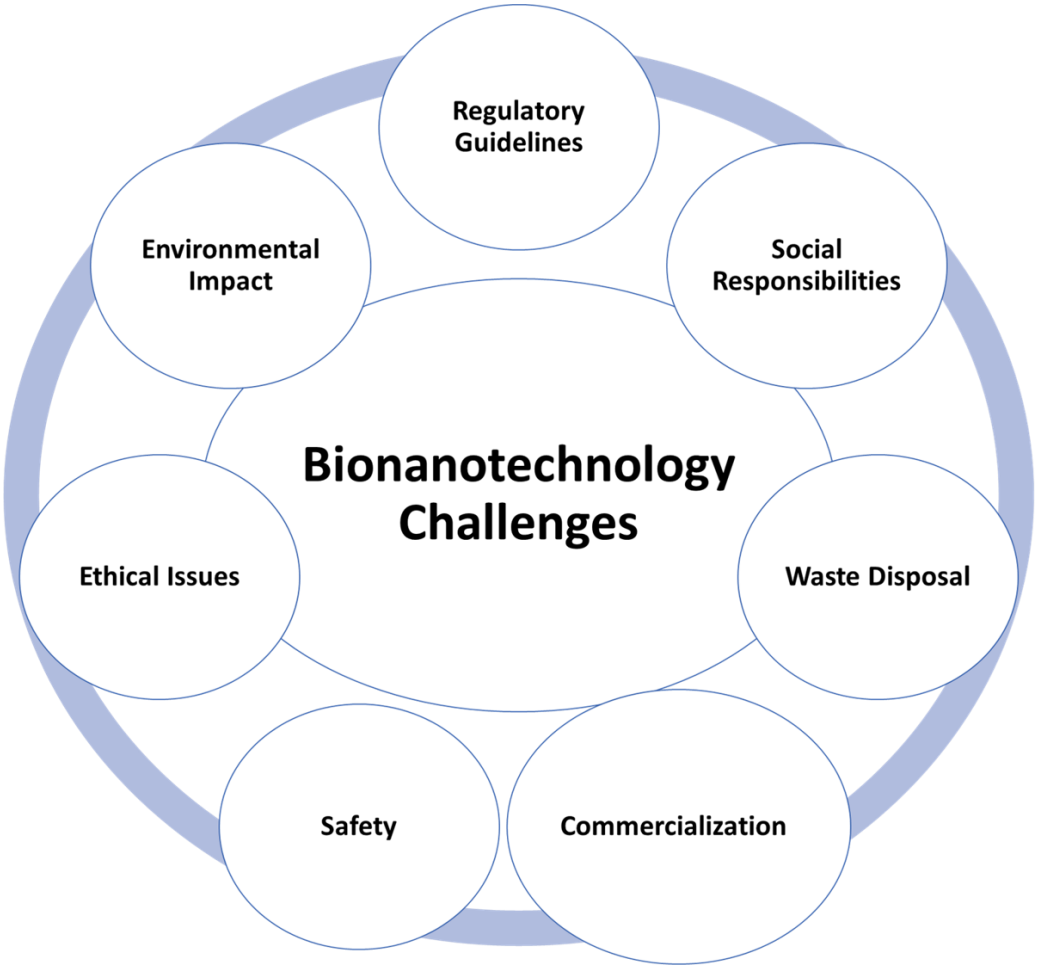

Fig. (4). The challenges in developing bionanotechnology-based products, ethical considerations, protection considerations, regulatory regulations, marketing, and environmental and social concerns.

POTENTIAL HAZARDS OF NANOPARTICLES

Nanoparticles have a potential danger comparable to particulate matter because of their extreme microscopic dimension, which has a particular benefit. Such particles may cause irregular respiratory, cardiovascular, and gastrointestinal pathologies. Intra-tracheal carbon nanotube instillation in mice has shown that carbohydrate tubes can lead to differing lung conditions, such as epithelial granuloma, interstitial inflammation, peribranchial inflammation, and lung necrosis. It has been observed that the toxicity of carbon nanotube is higher than that of black and quartz carbon. Nanomaterials have been reported to reach the

human body through different ports. Accidental or accidental touch is most likely to occur through the lungs during development or use, from which other vital organs may be quickly translocated through the bloodstream. Nanoparticles are capable of functioning as gene vectors at the cellular level. The central nervous system can be infiltrated directly by an olfactory axon or by systemic traffic through the olfactory bulb. Experiments of monkeys and rats found an aggregation by the olfactory direction of carbon and manganese nanoparticles. This shows that the transmission of nanoparticles could, in the future, provide an alternative route to bypass the blood-brain barrier. This can, however, also contribute to inflammatory reactions in the brain that need to be tested. Nanoparticular toxicity may also be extrapolated in the gastrointestinal tract, resulting in inflammatory bowel diseases. The toxicity of nanoparticles is attributed to the release of pro-inflammatory mediators that contribute to inflammatory response and organ injury. When swallowed, nanoparticles can enter the bloodstream, reach various systems and organs, and potentially cause toxicity. The human system results are difficult to extrapolate from experiments *in vitro* in animal models. They require more research and much-needed caution for human use.

CONCLUSION

The early days of nanobiotechnology are still. Nanobiotechnology's multilateral sector takes nearer and closer to existence the science of the almost incomprehensibly small unit. This growth's consequences are often so broad that nearly all science and technology areas would eventually be affected. Bionanotechnology opens the contemporary era's doors with great anticipation because of the uncertainties, controversies, and conflicts over bionanotechnology. Innovative pharmaceuticals, biomedicines, tissue engineering, gene delivery, bioremediation, genetic imaging, biomarkers, and biosensors will represent an essential landmark in the future. Moreover, nanobiotechnology will become an important method for diagnosing the target distribution of medicines and genes for intracellular purposes in the future.

A wide variety of applications in medicine is provided by nanobiotechnology. Innovations like pharmacy procurement schemes are just the beginnings of something new. Nanotechnology will treat many illnesses in the future that have no cure today. While the expectations from nanobiotechnology in medicine are high, the benefits are infinite. A thorough assessment of risk and safety considerations must be made in the use of nanotechnology in medical therapy. Researchers who reject nanotechnology accept that nanotechnology advances can proceed since it offers tremendous benefits, but research is essential to ensure

people's protection. Nanomedicine's role in treating human ailments and natural human physiology may be crucial in the future. It is also possible that if it operates smoothly, nanobiotechnology becomes an unavoidable part of life one day and saves countless lives.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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